CONTRIBUTIONS
from
BOYCE THOMPSON INSTITUTE

VOLUME 16
1950-1952

Published Quarterly by
BOYCE THOMPSON INSTITUTE FOR PLANT RESEARCH, INC.
Yonkers 3, New York

PUBLICATION COMMITTEE

Lawrence P. Miller, Chairman
Lela V. Barton
Bettie M. Brooks
A. E. Hitchcock

Lillian Teller and Mary R. Bateman, Librarians
Mary R. Bateman, Indexer
L. P. Flory and William G. Smith, Jr., Photographers

TABLE OF CONTENTS

VOLUME 16

No. 1

Issued April 13, 1950

Page

Sinnott, Edmund W. William Crocker—The man and scientist (January 27, 1874–February 11, 1950) 1

McCallan, S. E. A. Some chromate complexes and organic compounds as seed protectants 5

McCallan, S. E. A. Factors influencing spray injury by glyoxalidine derivatives to potted apple trees 21

McCallan, S. E. A. Factors influencing deposition in the vacuum bell jar duster 27

No. 2

Issued September 8, 1950

Flemion, Florence, and June Olson. *Lygus* bugs in relation to seed production and occurrence of embryoless seeds in various umbelliferous species 39

Hartzell, Albert, and Eleanor E. Storrs. Bioassay of insecticide spray residues in processed food 47

Barton, Lela V. Relation of different gases to the soaking injury of seeds 55

No. 3

Issued November 7, 1950

Miller, Lawrence P. Tolerance of sulfate-reducing bacteria to hydrogen sulfide 73

Miller, Lawrence P. Formation of metal sulfides through the activities of sulfate-reducing bacteria 85

Hitchcock, A. E., P. W. Zimmerman, Henry Kirkpatrick, Jr., and T. T. Earle. Growth and reproduction of water hyacinth and alligator weed and their control by means of 2,4-D 91

Burchfield, H. P., and George L. McNew. Mechanism of particle size effects of fungicides on plant protection 131

McNew, George L., and H. P. Burchfield. Particle size in relation to the fungitoxicity of dichloronaphthoquinone 163

CONTENTS

No. 4

ISSUED JANUARY 24, 1951

Page

ZIMMERMAN, P. W., and JULIE KJENNERUD. Flowering and other responses induced in *Piqueria trinervia* with photoperiodic treatment 177

KING, LAWRENCE J., J. A. LAMBRECH, and THOMAS P. FINN. Herbicidal properties of sodium 2,4-dichlorophenoxyethyl sulfate 191

No. 5

ISSUED APRIL 30, 1951

ZIMMERMAN, P. W., and A. E. HITCHCOCK. Growth-regulating effects of chlorosubstituted derivatives of benzoic acid 209

BARTON, LELA V., and JANET E. HOTCHKISS. Germination of seeds of *Eichhornia crassipes* Solms 215

ZIMMERMAN, P. W., and A. E. HITCHCOCK. Rose "sports" from adventitious buds 221

HITCHCOCK, A. E., and P. W. ZIMMERMAN. A quantitative method of measuring response of plants to growth regulators 225

No. 6

ISSUED JULY 11, 1951

CHANDLER, CLYDE. Flowering habits and fertility of some *Cinchona* species in Guatemala 249

SCHMITT, C. G. Comparison of a series of derivatives of 4,5-dimethyl-2-mercaptothiazole for fungicidal efficacy 261

KING, LAWRENCE J., and JOHN A. KRAMER, JR. Studies on the herbicidal properties and volatility of some polyethylene and polypropylene glycol esters of 2,4-D and 2,4,5-T 267

FLEMION, FLORENCE, and BETTY TRAVIS MACNEAR. Reduction of vegetative growth and seed yield in umbelliferous plants by *Lygus oblineatus* 279

FLEMION, FLORENCE, RICHARD M. WEED, and LAWRENCE P. MILLER. Deposition of P^{32} into host tissue through the oral secretions of *Lygus oblineatus* 285

No. 7

ISSUED NOVEMBER 24, 1951

WELLMAN, R. H. The economics of using fungicides 295

MCCALLAN, S. E. A. Testing techniques 299

TISDALE, W. H., and A. L. FLENNER. Derivatives of dithiocarbamic acid as fungicides 303

CONTENTS

Page

HORSFALL, JAMES G., and SAUL RICH. Fungitoxicity of heterocyclic nitrogen compounds 313
GRUENHAGEN, R. H., P. A. WOLF, and E. E. DUNN. Phenolic fungicides in agriculture and industry 349
MCNEW, GEORGE L., and HARRY P. BURCHFIELD. Fungitoxicity and biological activity of quinones 357
WHALEY, F. R., and J. B. HARRY. Chromate complexes as fungicides 375

No. 8

ISSUED FEBRUARY 20, 1952

BARTON, LELA V. Germination of seeds of *Juniperus virginiana* L. . . 387
BEALE, HELEN PURDY, and CAROLYN R. JONES. Virus diseases of tobacco mosaic and potato yellow dwarf not controlled by certain purified antibiotics 395

No. 9

ISSUED MAY 9, 1952

CARROLL, ROBERT B. Activation of sodium 2-(2,4-dichlorophenoxy)-ethyl sulfate 409
ZIMMERMAN, P. W., A. E. HITCHCOCK, and EDWARD A. PRILL. Substituted benzoic acids as growth regulators 419
FLEMION, FLORENCE, LAWRENCE P. MILLER, and RICHARD M. WEED. An estimate of the quantity of oral secretion deposited by *Lygus* when feeding on bean tissue 429
VLITOS, A. J. Biological activation of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate by *Bacillus cereus* var. *mycoides* 435

No. 10

ISSUED JULY 30, 1952

ZIMMERMAN, P. W., A. E. HITCHCOCK, and HENRY KIRKPATRICK, JR. Detection and determination of 2,4-D as a contaminant by biological methods 439
BACK, R. C. The insecticidal activity of the eta isomer of 1,2,3,4,5,6-hexachlorocyclohexane 451
HILCHEY, JOHN D., and ROBERT L. PATTON. Effects of irregular sex ratios on growth response data from nutritional assays upon *Blattella germanica* (L.) 455

CONTENTS

No. 11

ISSUED SEPTEMBER 22, 1952

Page

BARTON, LELA V., and A. GOENAGA. An electrical moisture meter for the determination of moisture in coffee beans 461

KING, LAWRENCE J. Germination and chemical control of the giant foxtail grass 469

Index 489

ERRATA

Vol. 16, page 1, line 12 from bottom, "spititual" should read "spiritual"

Vol. 16, page 5, paragraph 2, line 6, "than" should read "then"

Vol. 16, page 186, line 8 from top and line 12 from bottom, "primorida" should read "primordia"

Vol. 16, page 249, line 11, "Chicacacao" should read "Chicacao"

Vol. 16, page 271, line 1, "plants resulting" should read "plants, resulting"

Vol. 16, page 273, line 4, "was" should read "were"

Vol. 16, page 275, line 4 from bottom, "plantings" should read "planting"

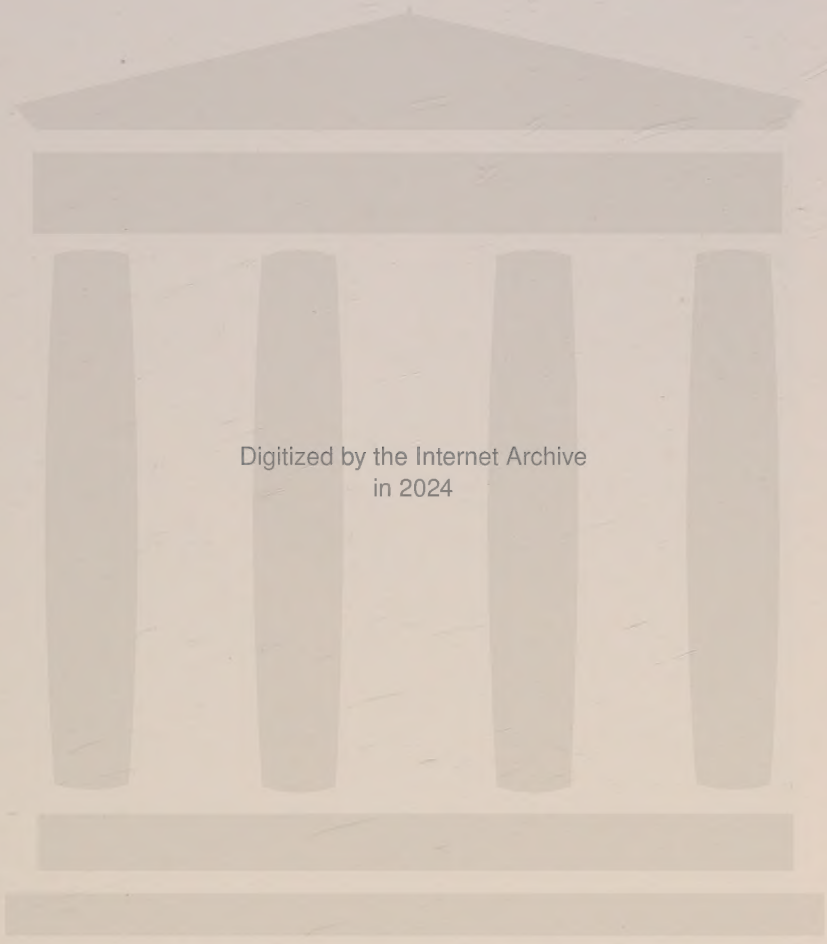
Vol. 16, page 280, line 6, "at" should read "an"

Vol. 16, page 296, footnote, "NcNew" should read "McNew"

Vol. 16, page 319, Table II, last line, "2,4,4-1 Trimethyl-2-oxazoline" should read "2,4,4-Trimethyl-2-oxazoline"

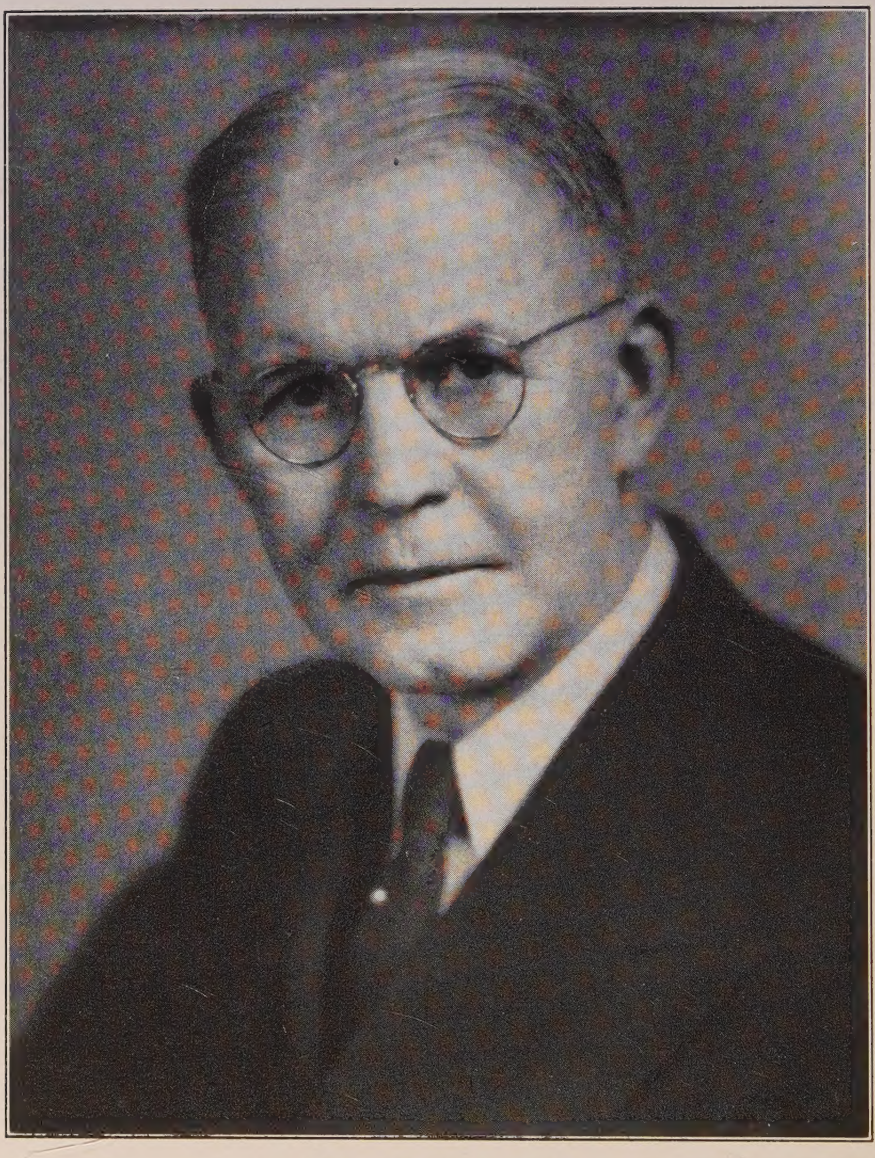
Vol. 16, page 325, Table III, line 6 from bottom, "-propyl-" should read "-propyl]-"

Vol. 16, page 330, line 12 from bottom, "1-(2-Cyanoethyl—L—pyroglutamic" should read "1-(2-Cyanoethyl)-L-pyroglutamic"



Digitized by the Internet Archive
in 2024

https://archive.org/details/boyce-thompson-institute-for-plant-research_1950-1952_16



WILLIAM CROCKER
1874–1950

WILLIAM CROCKER—THE MAN AND SCIENTIST[1]

(January 27, 1874—February 11, 1950)

EDMUND W. SINNOTT

In the death of Dr. Crocker, botanical science in the United States has lost one of its outstanding leaders. We may well say of him, "He was a man, take him for all in all, we ne'er shall look upon his like again." He was big—big in stature, in mind and in spirit—and in paying tribute to him we should consider his many-sidedness. He was a great botanist, but he was also very much more than that.

In the first place Dr. Crocker was a notable scientist and he would probably wish to be remembered thus than for anything else which he accomplished. A great scientist must have a number of qualifications which mark him off from ordinary folk, and these he possessed.

A scientist must be honest. The naked truth and nothing else must be his goal. He must not evade or cover up or tell half truths. Those who knew Dr. Crocker will testify how rigorously he maintained this attitude of complete honesty in his own work and how he demanded it in the work of every one at the Institute.

A scientist must be critical. One of his great qualities is that he must carefully evaluate all of his results by the methods which science has developed for testing its conclusions. This quality Dr. Crocker possessed in a high degree and there is probably no institution where the scientific product has always been scrutinized so rigorously and subjected to so much careful revision as at the Boyce Thompson Institute, as all who have published in its Contributions can testify.

A scientist must be an adventurer. The face of science is to the future and not the past, and its practitioners are spitiual descendents of the explorers of old. Such an explorer was Dr. Crocker, who pushed out across many frontiers and got a glimpse of the great possibilities ahead for plant research. He saw them not only in his own personal research but in that of his colleagues. An idea for a new program of investigation was always welcomed by him and was assured of a sympathetic hearing.

Dr. Crocker was far more than a scientific investigator, however. He was a great administrator and thus had to possess qualities which marked him out from men of science. A great administrator must have imagination, must see possibilities of scientific work and its contributions to society which often escape the eyes of other men. Dr. Crocker possessed such imagination in a high degree and it was he who first saw the possibilities

[1] Address delivered at the Memorial Service held at the Boyce Thompson Institute on February 17, 1950.

of such an institute as that over which he presided. He not only saw these, but had the ability to make others catch the picture of his vision and enthusiasm and thus make his dreams come true. The Institute, as we know it, is the outcome of the imaginative enthusiasm of its first Director.

A great administrator must also have optimism and faith to carry him through periods of discouragement and adversity. No great institution like this has smooth sailing all the time. There are ups and downs in its fortunes and it is essential to have in the man who directs it a firm hand on the tiller and a steady look ahead. We all can testify to Dr. Crocker's sure, steady and optimistic spirit in the long years in which he served as Director.

Such an administrator also must have, of course, executive ability in a high degree—the ability to bring things to pass, to persuade people to his point of view, to help his colleagues work together in harmony and efficiency and to maintain the material and financial fortunes of his insitution on a sound basis. These qualities Dr. Crocker possessed in high degree. He was not only a great leader but a good business man, with all that that implies; and the way in which the Institute has weathered the financial difficulties of the past twenty-five years is evidence of its Director's sound and solid judgment.

Quite apart from his accomplishments as scientist and administrator, however, Dr. Crocker was a great citizen of the community in which he lived, and of the nation. He took a very keen interest in the City of Yonkers and particularly in the development of its educational system, to the support of which he gave much time and energy over the years. Not only did he do this himself, but he continually encouraged the members of his staff to do their share of the business of the community as good citizens. This quality is much prized today when scientists are beginning to see that they must leave their ivory towers and take part in the affairs of society if our social order is to persist. One of the first men to see the importance of this was Dr. Crocker and he will long be remembered for these contributions by many people who knew little or nothing of his work as a scientist.

Today when we remember with gratitude what Dr. Crocker meant to all of us here we may well think of him not as a scientist or as an administrator or as a citizen, but primarily as a warm personal friend. He was one of the friendliest persons I have ever known. This characteristic was not a superficial one. He did not merely greet you pleasantly and courteously, but he really took an interest in your welfare. This showed itself in many ways, but most notably perhaps in the vigorous loyalty with which he supported all the members of the Institute staff in their problems and difficulties. He was never averse to a good fight if it was necessary and one could always count on him not to pussyfoot on any issue, but to go out vigorously in defense of persons and principles in which he believed. There

was never any question on which side of a controversy Dr. Crocker stood.

Despite our sense of loss in his passing we should be thankful that for so long he was able to direct this Institute, to which the best years of his life were given. Men leave various things behind them by which they are remembered, but in Dr. Crocker's case the Institute itself is his best memorial. One thinks of the inscription over Sir Christopher Wren's tomb in St. Paul's Cathedral: "Si monumentum requires, circumspice!" Thus we may well say of Dr. Crocker, "Look you for his monument, it is the Boyce Thompson Institute."

Dr. Crocker will be greatly missed by all of us, but time marches on and the old order inevitably changes. As I think of his passing, John Bunyan's words, in which he described how his hero passed over the River of Death, come to my mind: "So Valiant-for-Truth passed over and all the trumpets sounded for him on the other side."

SOME CHROMATE COMPLEXES AND ORGANIC COMPOUNDS AS SEED PROTECTANTS[1]

S. E. A. McCallan[2]

A method for evaluating chemicals as seed protectants by the use of peas in naturally infested greenhouse soil was described in 1948 (4). At that time the results with 157 chromate complexes were reported as an example of how the method works in practice. Recently an opportunity was presented to evaluate a larger series of chromate complexes and organic compounds. These materials which were kindly furnished by the Carbide & Carbon Chemicals Division, Union Carbide & Carbon Corporation, New York, N. Y., were selected on the basis of results with the slide-germination (1) and other tests. The number of compounds screened by the pea seed method now total 640 and it is the purpose of this paper to report on the more promising ones both as to greenhouse and field tests performed in 1949 in comparison with standard treatments.

INITIAL GREENHOUSE TESTS

This second series of tests was performed on 356 organic compounds and 127 chromate complexes. Most of these preparations were in dust form and could be tested readily. A few, some 40 odd, however, were liquids, pastes, waxes, or in other physical form which required formulation before testing. These were more or less satisfactorily formulated by dissolving or suspending in an equal amount of acetone, than grinding in a mortar with five or nine parts of Pyrax ABB dust (R. T. Vanderbilt Co., Inc., New York, N. Y.). All samples were first tested on pea (var. Perfection) at a dose of 0.0625 per cent (active ingredient) of seed weight in three replicated units of 10 seeds each as described previously (4). Only 35 compounds were considered promising after this first test. These were then tested on peas again at doses of 0.25, 0.0625, and 0.0156 per cent of seed weight replicated five times. The standards used here were thiram and Semesan (see p. 10) as well as the mercury-zinc-chromate (No. 224) and copper-zinc-chromate (No. 640) previously reported (4). Following this second test nine compounds remained of interest.

These nine compounds were then tested on beet (var. Detroit Dark Red), cantaloupe (var. Honey Rich), sweet corn (var. Ioana), cabbage (var. Penn State Ballhead), spinach (var. Giant Nobel), and lima bean

[1] A preliminary report was presented before the American Phytopathological Society, New York, N. Y. December, 1949 (5).

[2] The author wishes to express his indebtedness to Dr. C. S. Reddy, Iowa Agricultural Experiment Station, Ames, Iowa, and to Dr. J. D. Wilson, Ohio Agricultural Experiment Station, Wooster, Ohio, for generous permission to publish the results of their tests.

Copyright, 1950, by Boyce Thompson Institute for Plant Research, Inc.

(var. Henderson Bush) at doses of 1.0, 0.25, 0.0625, and 0.0156 per cent seed weight, except for beet which received four times these amounts. Two standard treatments considered especially appropriate for the crop in question were run as well as No. 224 and 640.

From these final (B.T.I.) greenhouse tests, including the replicated pea test, five experimental materials were considered the most promising and the percentage emergence, mean of all doses, is recorded in the second column of Tables I, II, and III for the various crops involved. Results with the standard treatments are also given.

EXPERIMENTAL FUNGICIDES

The five outstanding chemicals found in the greenhouse tests are:

No. 201 γ-Chloroacetoacetanilide, $ClCH_2COCH_2CONC_6H_5$

No. 854 Cupric γ-chloroacetoacetanilide

No. 5400 Reaction product of dimethyldithiocarbamate and sulfur dichloride

and the previously reported chromate complexes:

No. 224 $0.4HgO \cdot 3ZnO \cdot CrO_3$

No. 640 $CuO \cdot ZnO \cdot 1.3CrO_3 \cdot 5H_2O$

PHYSICAL AND CHEMICAL PROPERTIES

All of the chemicals tested could be ground to powders and were readily applied to the seeds as dust treatments.

The chromate complexes, No. 224 and 640, are neither true compounds nor simple mixtures but are "non-Daltonian in character, *i.e.*, distinct and reproducible chemical entities the ratios of whose components are capable of being varied continuously over a wide range of composition" (3, p. 209). The chromium is present in the hexavalent state and is of a low order of solubility. Empirical formulae are given above. It will be noted from the formula that No. 224 as now prepared and used has been modified and contains less mercury than the first sample (4). The Cr^{+6} solubility in 1.0 per cent suspension is less than 0.009 per cent. Like most chromate complexes, it possesses considerable color and would be described according to the Ridgway System (6) as "ochraceous buff." The copper-zinc-chromate complexes are described by Harry, *et al.*, (3). The Cr^{+6} solubility for No. 640 in 1.0 per cent suspension is less than 0.002 per cent and the color is "yellow green."

The γ-chloroacetoacetanilide (No. 201) melts at 137 to 138° C., and is relatively insoluble in water. The cupric salt of γ-chloroacetoacetanilide (No. 854) melts at 185 to 188° C. with decomposition. It also is relatively insoluble in water and would be characterized as "light Paris green" in color. Compound 5400, the reaction product of dimethyldithiocarbamate and sulfur dichloride, melts at 138° C., and is relatively insoluble in water.

MAMMALIAN TOXICOLOGY

Preliminary toxicological information has been obtained through the courtesy of the Chemical Hygiene Fellowship of the Mellon Institute of Industrial Research, University of Pittsburgh, Pittsburgh, Pa. It is emphasized that this information was obtained in screening tests. It is presented here to give a first approximation of toxicology.

The mercury-zinc-chromate (224) had an oral LD50 of 0.63 g. per kg. for male albino rats which is less toxic than that for some of the standard mercury and copper seed treatments. Similar tests for the copper-zinc-chromate (640) gave an LD50 of 3.62 g. per kg. which is less toxic than that for most standard seed treatments. In a special inhalation test of four hours' exposure to the dust, neither No. 224 nor 640 was harmful to mature rats. Several standard materials killed some or all of the rats in comparable tests. Skin irritation tests on the clipped rabbit belly with a 1 to 1 suspension in water provoked no response in the case of No. 640 and only a very mild one for No. 224 while of other common seed protectants tested, all seven produced more serious responses. Eye damage tests consisted in the application of the dry dust and also a 1 to 1 suspension in water in excess to the cornea of the rabbit eye. All nine seed treatments tested caused considerable corneal necrosis in both dry and wet application except No. 640 which caused only minor eye injury as a dry powder. Compound 224 rated intermediate in toxicity in the dry form and both No. 224 and 640 rated low in the injury scale for the suspension.

Compound 201, γ-chloroacetoacetanilide, has shown skin irritation to some research workers, and hence cannot be considered a practical seed treatment. Thus, No. 201 was not included in the winter Florida tests. The summer field tests, however, were initiated before this response had been clarified and these field data will be presented because of their general bearing on fungicidal action.

Cupric γ-chloroacetoacetanilide (No. 854) when fed to rats as a 10 per cent suspension in 0.25 per cent agar gave an LD50 of 1.87 g. per kg. The estimated LD50 for rabbits by skin penetration of suspensions in propylene glycol was 0.55 g. per kg. Ten per cent solutions or suspension in water, acetone, or methanol in a 24-hour test caused mild capillary infection on only a few of the rabbits, when applied to the clipped belly. In the four-hour rabbit belly irritation test the dry powder was less irritating than a water suspension. Human skin patch tests are in progress.

Compound 5400 (reaction product of dimethyldithiocarbamate and sulfur dichloride) in single dose oral toxicity tests to rats showed an LD50 of 0.30 g. per kg. Indications are that in propylene glycol the LD50 by skin adsorption in rabbits is above 2.0 g. per kg.

TABLE I

PERCENTAGE EMERGENCE IN SEED TREATMENT TESTS AT DIFFERENT STATIONS; DOSE IN OZ. PER 100 LB. RECORDED UNDER CROP NAME

Material	Green-house B.T.I.	Yonkers, N. Y.		Bridgeton, N. J.		Wooster, Ohio		Willard, Ohio		Belle Glade, Fla.	Station mean	Mean omit. Green-house & Fla.
	Pea											
	a	2	.5	2	.5	3.6	1.8	3.6	1.8	2		
640	67	91	89	89	71	81	79	85	88	57	76.8	84.1
224	75	92	86	81	67	85	80	90	83	41	74.7	83.0
854	70	94	91	88	70	72	73	82	80	48	73.8	81.2
Arasan	58	90	87	76	61	80		85		34	69.0	80.5
5400	65	92	86	75	57	71	77	77	77	32	67.2	76.5
201	74	95	92	86	69	79	72	91	87			83.9
Spergon		92	89	82	53	80		83				80.3
Phygon		90	89	87	81							
Semesan	81	91	92	90	79							
Y. Cuproc.		91	84	84	62							
Control	26	82		42		44		64		12	45.0	58.0
LSD 5%	12	4		8		3		3		15		
	Lima bean											
	b	2	.5	2	.5	3.6	1.8			2		
5400	77	84	85	81	68	85	91			10.0	66.8	82.3
Arasan	81	86	83	72	77	83				2.8	65.2	80.7
Spergon	76	82	84	79	68	85				1.8	63.9	80.5
854	76	74	71	72	75	77	80			2.2	60.5	74.8
640	49	11	81	27	78	64	77			1.4	43.9	56.3
201	74	46	73	54	67	85	82					67.8
Semesan		80	85	82	83							
Phygon		78	78	84	61							
Y. Cuproc.		16	80	28	71							
224	48					34	61					
Control	61	86		29		77				0.2	50.6	64.0
LSD 5%	10	10		9		4				1.9		
	Spinach											
	b	4	1	4	1	6	3	6	3	4		
854	61	2.8	2.4	50	42	30	36	36	41	13	32.4	30.0
5400	51	2.2	2.6	51	40	34	39	37	45	13	31.6	31.3
Arasan	52	1.2	4.0	45	36	40		43		8	31.0	31.5
640	53	1.0	0.4	45	22	39	41	30	48	12	29.7	28.3
224	50	2.0	0.6	45	20	44	38	43	46	1	28.3	29.8
201	56	1.4	3.0	44	43	36	37	48	43			31.9
Spergon		1.2	1.0	14	7	31		49				22.9
Y. Cuproc.	57	3.0	3.8	55	48							
Phygon		3.0	3.6	60	42							
Semesan		1.8	2.6	48	32							
Control	40	1.8		17		16		30		1	17.6	16.2
LSD 5%	No	No		13		3		4		5		
	Beet											
	c	8	2	8	2	10	5	10	5	8		
854	138	4.2	1.6	34	38	75	57	84	87	63	65.2	47.6
Arasan	134	6.6	3.8	62	42	78		97		7	62.2	58.1
5400	131	1.6	5.8	49	31	82	68	89	91	32	62.0	52.2
640	125	3.4	0.6	41	20	72	70	84	93	22	56.5	48.0
224	109	1.2	0.2	20	26	49	47	79	88	5	44.9	38.8
201	126	4.2	0.4	29	27	75	58	95	79			46.0
Spergon		1.0	0.6	22	20	40		84				36.5
Phygon		5.2	3.6	36	65							
Semesan		1.4	1.8	38	37							
Y. Cuproc.	116	2.0	3.0	43	28							
Control	102	1.6		26		44		76		5	42.4	36.9
LSD 5%	23	4.0		18		5		6		8		

a. Mean of 4, 1, 0.25 oz.; *b*. mean of 16, 4, 1, 0.25 oz.; *c*. mean of 64, 16, 4, 1 oz.

TABLE II

PERCENTAGE EMERGENCE IN SEED TREATMENT TESTS AT DIFFERENT STATIONS; DOSE IN OZ. PER 100 LB. RECORDED UNDER CROP NAME

Material	Greenhouse B.T.I.	Yonkers, N. Y.		Bridgeton, N. J.		Belle Glade, Florida	Station mean
	Cucurbit						
	a	2	.5	2	.5		
201	85	17	23	61	59		55.0
5400	84	20	19	58	55		53.3
854	81	15	19	55	55		50.1
Spergon	70	24	15	57	48		47.3
Arasan	72	19	16	44	47		45.0
640	69	18	8	46	41		41.8
224	74	12	12	34	32		39.7
Phygon		21	17	60	44		
Y. Cuprocide		21	10	50	51		
Semesan		18	9	42	51		
Control	48	12		32			30.7
LSD 5%	4	10		5			
	Cabbage						
	a	4	1	4	1		
Arasan	59	30	22	63	50		47.2
224	63	30	20	51	55		47.0
Semesan	58	31*b*	29*c*	48*b*	49*c*		45.5
5400	57	29	26	52	41		43.7
854	48	33	17	46	54		41.0
201	53	20	18	31	42		36.2
640	50	28	21	33	34		36.0
Phygon		22	27	44	47		
Spergon		22	17	38	40		
Y. Cuprocide		8	17	33	33		
Control	36	15		32			27.7
LSD 5%	10	No		15			
	Snap bean						
		2	.5	2	.5	2	
Arasan		85	85	78	77	55	72.5
5400		88	82	79	79	52	72.0
640		84	85	79	78	50	71.0
854		81	84	78	85	46	70.0
224		85	80	80	73	51	70.0
201		85	88	78	81		
Semesan		85	86	80	77		
Phygon		85	83	82	76		
Spergon		83	82	77	77		
Y. Cuprocide		78	86	78	74		
Control		83				33	
LSD 5%		5		No		No	
	Tomato						
		4	1	4	1	4	
640		20	19	68	66	67	51.2
854		24	20	64	60	51	45.0
5400		19	22	61	71	44	43.5
Arasan		21	15	60	62	47	42.0
224		15	15	54	58	33	34.7
Phygon		15	21	70	58		
Semesan		16	22	61	63		
Y. Cuprocide		9	15	62	58		
Spergon		19	13	59	51		
201		18	11	52	52		
Control		15		52		37	34.7
LSD 5%		No		No		16	

a. Mean of 16, 4, 1, 0.25 oz.; *b*. 6 oz.; *c*. 1.5 oz.

FIELD TESTS

Field tests have been performed on the five above experimental compounds in comparison with appropriate standards at seven stations in five different states on various crops.

NEW YORK

Ten crops tested at Yonkers, New York, were as follows: pea (*Pisum sativum* L.) var. Thos. Laxton, beet (*Beta vulgaris* L.) var. Detroit Dark Red, cabbage (*Brassica oleracea* L.) var. Golden Acre, spinach (*Spinacia oleracea* L.) var. Bloomsdale Savoy, sweet corn (*Zea mays* L.) var. Golden Cross Bantam, lima bean (*Phaseolus limensis* Macf.) var. Henderson Bush, muskmelon (*Cucumis melo* L.) var. Bender Surprise, snap bean (*Phaseolus vulgaris* L.) var. Tendergreen, tomato (*Lycopersicon esculentum* Mill.) var. Bonny Best, peanut (*Arachis hypogaea* L.) var. Spanish.

The rate of treatment was 2 oz. per 100 lb. on the larger seeds for the experimental materials; 4 oz. per 100 lb. for cabbage, spinach, and tomato; and 8 oz. per 100 lb. for beets. The standard fungicides were applied according to manufacturers' directions when given, and varied from 2 to 24 oz. per 100 lb., the details of which are stated in the tables. Thus, the doses of the experimental materials applied were equal to or less than the standard materials and hence in some cases the former were under a dosage handicap. In addition, in order to emphasize difference, all treatments were applied at one-quarter of the above rates.

The standard protectants employed were thiram, *i.e.*, Arasan (50 per cent tetramethylthiuram disulfide) and Semesan (30 per cent hydroxymercurichlorophenol), duPont Semesan Co., Wilmington, Del., chloranil, *i.e.*, Spergon (96 per cent tetrachloro-*p*-benzoquinone), and Phygon (98 per cent 2,3-dichloro-1,4-naphthoquinone), U. S. Rubber Co., New York, N. Y., and cuprous oxide, *i.e.*, Yellow Cuprocide (86 per cent Cu), Röhm & Haas Inc., Philadelphia, Pa.

The seeds were treated on the rolling machine (4) and counted out into replicate lots of 100. Five replicates of each treatment, each dose, and each kind of seed were planted on the Institute farm at Yonkers. The small seeds (beet, cabbage, spinach, and tomato) were in units of three-foot rows and the large seeds in units of six-foot rows. A replicate block consisted of seven rows across and three units lengthwise thus giving 21 treatments, *i.e.*, ten chemicals × two doses + control. The five replicate blocks gave a total of 1,000 seeds per chemical. The soil is Merrimac loam and the seeds were planted at normal time to several weeks ahead of normal for this area. In order to accentuate seed decay the seed was covered somewhat deeper than normal, in addition to the earlier planting. This probably resulted in the very poor stands reported for some crops. Results on per-

centage emergence were taken about three weeks after planting and are recorded for both doses in column three of Tables I, II, and III.

NEW JERSEY

A similar series of tests were performed at Bridgeton, N.J., through the courtesy of the Seabrook Farming Corporation who furnished the land. An additional set of five replicates was planted at Bridgeton and all crops and treatments were included except peanuts. The plot design was also similar. The soil here is Sassafras sandy loam; the seed was planted on May 9 and emergence data recorded on June 1 and 2. Considerable rain fell between planting and record taking dates. The emergence data for both doses are recorded in column four of Tables I, II, and III.

TABLE III

Percentage Emergence in Corn Seed Treatment Tests at Different Stations

Material	Greenhouse B.T.I.	Yonkers, N. Y.		Bridgeton, N. J.		Wooster, Ohio		Willard, Ohio		Greenhouse Ames, Iowa		Belle Glade, Fla.	Station mean	Mean omit. Fla.
	Dose—oz. per 100 lb.													
	[a]	2	.5	2	.5	3.6	1.8	3.6	1.8	3.6	1.8	2		
854	79	93	95	82	86	87	84	73	71	89	90	83	83.9	84.0
Arasan	77	94	93	88	78	86		83		90[b]	88[c]	60	81.6	85.2
5400	73	94	94	83	79	83	88	78	70	91	88	66	80.4	82.8
224	84	94	91	89	76	89	86	73	67	92[d]	90	54	80.2	84.6
640	67	92	92	82	77	82	85	72	64	81		51	74.6	78.5
201	76	92	90	86	83	81	85	84	67		93			83.8
Spergon	56	91	89	80	71	82		74		86[d]				77.3
Semesan		92	93	90	81									
Phygon		91	93	82	89					90[e]	89[f]			
Y. Cuproc.		80	88	79	79									
Control	42	90		77		76		88		65		17	65.0	73.0
LSD 5%	9	4		7		2		4		4		6		

[a] Mean of 16, 4, 1, 0.25.
[b] Arasan SF 1.2 oz. per 100 lb.
[c] Arasan SF 0.9 oz. per 100 lb.
[d] 2.7 oz. per 100 lb.
[e] Phygon paste 1.8 oz. per 100 lb.
[f] Phygon paste 0.9 oz. per 100 lb.

OHIO

The experimental seed treatments were tested at Wooster and at Willard, Ohio, by Dr. J. D. Wilson. The crops treated were pea (var. Freezonian), beet (var. Detroit Dark Red), spinach (var. Bloomsdale Long Standing), lima bean (var. Fordhook 242), and sweet corn (var. Golden Cross Bantam). All experimental treatments were applied at 1 and 2 oz. per bushel (converted in tables to 100 lb. basis). The standards were thiram and chloranil at 2 oz. per bushel. Thus, here again the experimental materials were under a slight dosage handicap. The soil at Willard is muck and the lima beans were omitted. Seed from all treatments and doses were planted in five replicate lots of 100 seeds each. The emergence data for both doses are given in column five of Tables I and III for the Wooster results and column six of Tables I and III for the Willard results.

IOWA

Tests in Iowa on hybrid field corn were conducted by Dr. C. S. Reddy. All experimental materials in 1949 were first tested in the greenhouse preceded by a cold treatment in comparison with various standard fungicides. The mean results of nine such tests are reported in column seven of Table III. The same materials were then tested in replicated field plots at Cones-

TABLE IV

EFFECT OF VARIOUS SEED TREATMENTS OF CORN ON YIELDS IN BUSHELS PER ACRE AT THREE LOCATIONS IN IOWA (DATA OF C. S. REDDY)

Materials	Oz. per 100 lb.	Conesville	Kanawha	Shenandoah	Mean
		1947			
224	1.3	54	69		61.5
Phygon	0.9	50	72		61.0
Arasan SF	0.9–1.8	48	71		59.5
Control		36	71		53.5
		1948			
224	2.7	81	97	103	93.7
Spergon	2.7	79	100	98	92.3
Phygon	0.9	72	100	99	90.3
Arasan SF	0.9	73	94	97	88.0
224	1.3	73	92	83	82.7
Control		65	91	80	78.7
		1949			
5400	3.6	55	96		75.5
224	2.7	54	93		73.5
854	1.8	52	92		72.0
201	1.8	51	91		71.0
Arasan SF	1.2	51	91		71.0
Arasan SF	0.8	53	89		71.0
Phygon paste	1.8	50	92		71.0
Spergon	2.7	56	85		70.5
5400	1.8	51	90		70.5
224	1.8	51	89		70.0
Phygon paste	0.9	50	90		70.0
640	3.6	52	88		70.0
854	3.6	50	88		69.0
Control		48	87		67.5

ville and Kanawha and the results are given in Table IV as yields per acre. In addition, the mercury-zinc-chromate (224) had been field tested at the two above stations in 1947, and at the same two and Shenandoah in 1948. These data are also given in Table IV. All results for Iowa reported here are on the regular treatments unmodified by the addition of stickers. The doses applied in the Iowa tests varied and are given in the tables. In this case, contrary to all others, the standard treatments were applied at equal or smaller doses and hence here have a slight dosage handicap.

FLORIDA

By means of the facilities of Dr. G. R. Townsend, four experimental compounds, No. 224, 640, 854, and 5400, were compared with standard treatments for nine crops at Belle Glade, Florida. The seed furnished by Dr. Townsend was treated in Yonkers and consisted of the following: pea var. Little Marvel), beet (var. Detroit Dark Red), spinach (var. Bloomsdale), snap bean (var. Stringless Black Valentine), lima bean (var. Fordhook 242), sweet corn (var. Golden Cross Bantam), peanut (var. Spanish Runner), and delinted cotton. The dose applied was 2 oz. per 100 lb. for all seed except spinach and tomatoes which received 4 oz. and beets which received 8 oz. The standard treatment was thiram for all crops except cotton where Ceresan (ethylmercury phosphate 5 per cent, duPont Semesan Co., Inc.) was used. In the case of lima bean, the mercury-zinc-chromate was omitted because of its now well established injurious effect and chloranil was substituted. Five replicate lots of 100 seeds each were planted on the muck at Belle Glade during mid-November 1949 and emergence data recorded several weeks later. Due to unusually cold weather, the cold-sensitive lima beans, peanuts, and cotton did not produce a good stand. Results for all crops except peanuts and cotton, which were too meager and inconclusive, are given in column seven of Table I, column five of Table II, and column eight of Table III.

DISCUSSION OF RESULTS

The results for all treatments, crops, and stations may be seen in Tables I, II, and III. In order to conserve tabular space the original percentage emergence figures, for the most part, have been rounded to the nearest whole number. Least significant differences at the 5 per cent level (7) have been calculated for all data except the field tests from Iowa. In most cases significant differences were found between the treatments for any given crop at any station. The notable exceptions are snap beans and tomatoes (Table II). From the percentage emergence data provided in these tables, detailed comparisons may be made of the relative performance of the various seed treatments at any given station. Of more general interest, however, are the average results or overall picture for the different treatments at all stations for a given crop. In spite of the fact that at some stations and for some fungicides there was only one dose, the most informative average appears to be the "station mean," that is, the mean of the station means. Hence the station means have been calculated for all fungicides that were tested at all stations and are shown in the second last column of Tables I and III, and the last column of Table II. The standard treatment, chloranil, is of considerable interest but this was not tested in Florida or in the Institute greenhouse for most crops; likewise compound No. 201 was omitted in Florida. Thus, in order to compare

these treatments with the others, a second column of station means omitting the Florida and/or greenhouse data has been calculated for peas, beets, spinach, lima beans, and corn, and is shown in Tables I and III.

RANKED DATA

It is desirable to combine the data for all stations for a given crop and apply a test of significance of difference in order to draw general conclusions as to performance. However, due to the wide variation in response level at the different stations, the variances are not homogeneous and hence the data cannot logically be combined for an over-all analysis of variance (7). By employing the method of ranks, the requirements for homogeneity of variances and a normal distribution are not needed, and a simple and rapid test may be applied with but little loss in efficiency (2). In addi-

TABLE V

MEAN RANKS AND SIGNIFICANT GROUPINGS FOR DIFFERENT FUNGICIDES ON THE SEEDS OF VARIOUS CROPS

Pea		Beet		Spinach		Lima bean		Tomato	
640	1.8	Thiram	1.8			5400	1.6		
224	2.2	5400	2.0	854	2.5	Thiram	2.0	640	1.7
854	2.7			Thiram	2.7			854	2.0
		854	2.7	5400	2.9	Chloranil	3.4	5400	2.7
Thiram	3.9	640	3.5	640	3.6	854	3.8	Thiram	3.7
5400	4.4			224	3.8				
		224	5.4			Control	4.6	224	5.5
Control	6.0	Control	5.6	Control	5.6	640	5.6	Control	5.5

Cabbage		Snap bean		Cucurbit		Corn	
Thiram	2.0			201	1.0	5400	3.1
224	2.5			5400	2.2	224	3.2
Semesan	2.7					Thiram	3.3
5400	3.7			854	3.7	854	3.6
854	4.8			Chloranil	4.2	201	3.9
		5400	1.8	Thiram	4.7		
201	6.0	Thiram	2.2	224	6.2	640	5.9
640	6.3	640	3.3	640	6.3	Chloranil	6.3
		854	3.5				
Control	8.0	224	4.2	Control	7.8	Control	6.9

tion, different kinds of data, such as percentage emergence and acre yields, may be combined. Accordingly all treatments tested at all stations for a given crop have been ranked by station average and the mean ranks are shown in Table V.

By using the χ_r^2 test as described by Wilcoxon (8) the data have been separated into significantly different groups which are separated by a horizontal space in Table V. For example, for peas No. 640, 224, and 854 are significantly better (19:1) than thiram and 5400, which in turn are significantly better than the control. Thus, at a glance all treatments may be compared and their relative performance noted.

RANK CORRELATIONS

It is of some concern to correlate the relative performance of the treatments at different stations; this also may be done on the ranked data as illustrated by Wilcoxon (8). Correlations between various stations' results deemed of interest have been calculated and are recorded in Table VI. It is to be emphasized that in preparing the rank correlations all data have been utilized and not necessarily only those which are given in Table V. For example, in correlating Yonkers and Bridgeton, 11 different treatments were available, and in comparing Wooster and Willard, 8 treatments

TABLE VI

Significance of Rank Correlations

Comparison			Corn	Pea	Beet	Spinach	Lima bean	Cucurbit	Cabbage	Snap bean	Tomato
		Yonkers	No	No	Sign.	No	Sign.*	No	No		
		Bridgeton	Sign.	Sign.	High	Sign.	High	Sign.	Sign.		
B.T.I.		Wooster	High	No	No	No	Sign.				
green-	vs.	Willard	No	Sign.	No	No					
house		Belle Glade	No	No	No	Sign.	Sign.				
		Ames greenhouse	Sign.								
		Yonkers	Sign.	No	No	No	No			No	No
Belle	vs.	Bridgeton	Sign.	High	No	Sign.	Sign.			No	No
Glade		Wooster	No	No	No	No	No				
		Willard	No	No	No	No					
Yonkers	vs.	Bridgeton	No	No	High	High	High*	High	High	No	High
Wooster	vs.	Willard	No	No	Sign.	No					
Conesville	vs.	Kanawha	No								

* Omitting control.

were used. Of primary interest is the correlation between the results at the Institute greenhouse where the initial testing was done and the results at the various field stations. Outstanding here is the significant or highly significant correlation with Bridgeton for all crops. With each of the other stations the results were significantly correlated with somewhat less than half the crops. A better correlation between the greenhouse and Yonkers field tests might have been expected; a possible explanation is the unusual depth of planting in the field. In the case of lima beans there was significant correlation at all stations. Altogether the correlation in order of ranking between the initial Institute greenhouse tests and the various field stations was significant or highly significant in half the cases. This gives added confidence to the greenhouse screening methods (4).

Florida is being used as a winter trial ground for new fungicides; hence the correlations between Florida and the northern vegetable stations have been determined. The Bridgeton correlations are significant for four of the seven crops, but for most all other crops and stations there was no significant correlation. This perhaps is to be expected because of the wide difference in environment.

It is of some interest to compare the naturally paired field stations of Yonkers and Bridgeton in the East, Wooster and Willard in Ohio, and

Conesville and Kanawha in Iowa. The correlation between Yonkers and Bridgeton is surprisingly good, being highly significant for six out of nine crops. Wooster and Willard, however, showed a significance for only one crop, beets, out of a total of four crops. A possible explanation here is the marked difference in soil types. The field corn results in Iowa were not significantly correlated between the two field stations. However, the greenhouse results were significantly correlated with those of the Institute greenhouse on sweet corn.

Lack of correlation is often due to the erratic response of one or two fungicides, as in Iowa. Also it should be pointed out that the number of paired fungicides is not large and it is thus difficult to attain significance. Even with the expected lack of homogeneity between the various stations it is still possible to show the more important overall significant differences between various of the fungicides as has been done in Table V.

GREENHOUSE TESTS ON SORGHUM

Preliminary greenhouse tests have been made at the Institute on sorghum (*Holcus sorghum* L.) seed treated with the experimental fungicides. The seed which had a somewhat lowered vitality, especially suitable for fungicide evaluation, was kindly furnished by the Associated Seed Growers Inc., New Haven, Conn. There were two varieties, Hegari and Martin's Combine. The seed was treated at rates of 4 and 1 oz. per 100 lb. Fifty seeds were planted for each dose of each fungicide in infested greenhouse soil. The mean percentage emergence in comparison with Arasan and Spergon is given in Table VII.

TABLE VII

PERCENTAGE EMERGENCE OF SORGHUM IN GREENHOUSE SEED TREATMENT TESTS. MEAN DOSE OF 4 AND 1 OZ. PER 100 LB.

Material	Hegari	Martin's Combine	Mean
224	78	86	82
854	73	71	72
640	66	76	71
5400	67	61	64
Arasan	55	73	64
Spergon	51	57	54
Control	28	16	22
LSD 5%	14	12	9

It will be seen that the mercury-zinc-chromate (224) gave the best results and was significantly ahead of the standard materials for both varieties. In the mean results it was even significantly better than the next best compounds, cupric γ-chloroacetoacetanilide (854) and the copper-zinc-chromate (640).

SEED STORAGE

A limited number of treated seeds of pea, beet, spinach, sweet corn, lima bean, cantaloupe, and cabbage held in storage at room temperature were available for viability tests. The seeds had been treated with varying doses, the highest of which was about four times as strong as the recommended doses, and they had been held for 10 to 12 months in lightly stoppered bottles. Germination tests in steamed and infested greenhouse soil indicated for the most part that the treated seed had not lost its viability or efficiency relative to standard seed protectants. There were, however, exceptions; thus, the mercury-zinc-chromate significantly decreased the germination of beet, cabbage, and cantaloupe at the highest dose and the copper-zinc-chromate decreased that of beet and cabbage. Also the chromate complexes at higher doses adversely affected the germination of lima bean as would be expected. It is to be noted that in most cases where the experimental treatments had performed well in the control of seed decay they did not adversely affect the germination of the treated seed after storage.

CONCLUSIONS

In order to simplify the drawing of general conclusions as to seed treatment value, the rank data of Table V have been summarized in Table

TABLE VIII

Summary of Seed Treatment Ratings

Crop	224	640	201	854	5400	Thiram	Chloranil
Peas	A	A	A	A	B	B	B
Spinach	B	B	B	B	B	B	B
Beets	C	B	B	B	A	A	C
Limas	D	C	C	B	A	A	B
Cucurbits	B	B	A	B	A	B	B
Cabbage	A	B	B	A	A	A	B
Beans	C	C	C	C	C	C	C
Tomatoes	C	B	C	B	B	B	C
Corn	A	B	A	A	A	A	B

VIII. Here the treatments have been accorded an A, B, C, or D rating; C is assigned to all treatments which are not significantly different from the check; B is the group significantly better than the check; and A the most promising group which is significantly better than B; D rating is for treatments significantly poorer than the check, *i.e.*, injurious. The standard treatments thiram and chloranil have been included; sufficient data were not available for other standards. It will be seen that for many crops one or more of the experimental compounds was found at least equal to the best standard treatment and in some cases they are significantly better. Results with peanuts and cotton were too limited, due to poor stands in-

duced by local conditions, to arrive at any conclusions for any of the treatments. The sorghum results have not been included formally in the summary table because of their preliminary nature.

The mercury-zinc-chromate (224) is especially promising on pea, cabbage, and corn; fair results were obtained on spinach and cucurbits. It is decidedly injurious to lima bean and probably without effect on beet, snap bean, and tomato. Results with corn have been most interesting and encouraging. On the basis of rank data, No. 224 placed high for the mean of all stations, being exceeded only by No. 5400. It has also led all standard treatments in all three years of Iowa yield tests. There is indication that its performance on muck soil (Willard, Ohio, and Belle Glade, Florida) is less effective. The higher dose for corn of 1½oz. per bushel or about 3 oz. per 100 lb. appears to be necessary for best performance. Indications are that the mercury-zinc-chromate will show to best advantage under severe conditions of unfavorable soil and temperature. The preliminary sorghum results also indicate that the mercury-zinc-chromate may have a very promising application on this crop.

The copper-zinc-chromate (640) gave best results on peas. Here its average results were significantly better than chloranil or thiram, generally considered outstanding for this crop. Good results were obtained on sorghum and fair ones on beet, spinach, cucurbit, cabbage, and tomato. Actually the copper-zinc-chromate was first on tomato though none of the treatments were outstanding. It may be injurious to lima bean.

Compound 5400, reaction product of dimethyldithiocarbamate and sulfur dichloride, is probably the most generally effective of all the experimental fungicides. It is especially promising on lima bean where it placed first in three stations and second in the remaining two. It is also promising on corn where it ranked first, and on beet, cabbage, and cucurbits. It was the only treatment exclusive of No. 201 to rate an A on cucurbits. Fair results were obtained on pea, spinach, and tomato. Like all other treatments including standards, no difference could be shown over the checks for snap bean.

Cupric γ-chloroacetoacetanilide (No. 854) was very effective on pea, corn, and cabbage and gave fair results on the other crops reported.

γ-Chloroacetoacetanilide (No. 201), although giving excellent results in seed protection on pea, cucurbit, and corn, cannot be considered a practical treatment because of skin irritation to operators.

Since one or more of all four experimental fungicides have given promising data on all of the ten crops reported above (excepting snap bean where even the standard treatments did not show to advantage), it is probable that they would also give encouraging results as seed treatments on a variety of other crops.

SUMMARY

From a series of 640 chromate or organic compounds evaluated as possible seed treatments by greenhouse tests on peas and other seeds, there were selected as promising seed protectants the following four compounds: mercury-zinc-chromate complex, $0.4HgO.3ZnO.CrO_3$ (No. 224); copper-zinc-chromate complex, $4CuO.Zno.1.3CrO_3.5H_2O$ (No. 640); cupric γ-chloroacetoacetanilide, cupric salt of $ClCH_2COCH_2CONC_6H_5$ (No. 854); and the reaction product of dimethyldithiocarbamate and sulfur dichloride (No. 5400). A fifth compound, γ-chloroacetoacetanilide (No. 201), also gave good protection, but was later eliminated because of skin irritation to humans.

Limited storage tests indicate no reduction in the effectiveness of the chemical or deleterious effect on the seed of those crops on which they gave good field results. Preliminary toxicological tests on animals indicate no unusual health hazards.

These experimental compounds were tested under field conditions in comparison with standard protectants, especially thiram and chloranil, on pea, spinach, beet, lima bean, cucurbit, cabbage, snap bean, tomato, and corn. Field tests on some or all crops were performed in 1949 at seven different stations representing New York, New Jersey, Ohio, Iowa, and Florida. Greenhouse tests were made on sorghum.

The mercury-zinc-chromate was the outstanding material tested on corn, ranking high for mean of all stations; in addition, it placed ahead of the standard treatments in each of three years' yield tests in Iowa. Good results were also obtained on pea, cabbage, and sorghum and fair results on spinach and cucurbit. However, it is decidedly injurious to lima bean and probably ineffective on beet, snap bean, and tomato.

The copper-zinc-chromate ranked first on pea. Fair results were obtained on the other crops excepting lima bean to which it is probably injurious.

Cupric γ-chloroacetoacetanilide is effective for pea, corn, cabbage, and sorghum and fair for the other crops.

The reaction product of dimethyldithiocarbamate and sulfur dichloride (No. 5400) ranked first on lima bean, corn, and cabbage and also was good on beet and cucurbit and fair on the other crops. It is the most generally effective of all the compounds tested.

Note Added in Galley Proof

Preliminary data received on the human skin patch tests with No. 854 noted on p. 7 indicate that the irritation is sufficient to preclude the practical use of this material as a seed treatment.

LITERATURE CITED

1. AMERICAN PHYTOPATHOLOGICAL SOCIETY. COMMITTEE ON STANDARDIZATION OF FUNGICIDAL TESTS. The slide-germination method of evaluating protectant fungicides. Phytopath. **33**: 627–632. 1943.
2. FRIEDMAN, MILTON. The use of ranks to avoid the assumption of normality implicit in the analysis of variance. Jour. Amer. Statist. Assoc. **32**: 675–701. 1937.
3. HARRY, J. B., R. H. WELLMAN, F. R. WHALEY, H. W. THURSTON, JR., and W. A. CHANDLER. Development of copper-zinc-chromate complexes as potato fungicides. Contrib. Boyce Thompson Inst. **15**: 195–210. 1948.
4. MCCALLAN, S. E. A. Evaluation of chemicals as seed protectants by greenhouse tests with peas and other seed. Contrib. Boyce Thompson Inst. **15**: 91–117. 1948.
5. ———. Organic and chromate compounds as seed treatments. Phytopath. **40**: 18. 1950.
6. RIDGWAY, ROBERT. Color standards and color nomenclature. 44 pp. Washington, D. C. 1912.
7. SNEDECOR, GEORGE W. Statistical methods. 4th ed. 485 pp. Iowa State College Press, Ames, Iowa, 1946.
8. WILCOXON, FRANK. Some rapid approximate statistical procedures. 12 pp. American Cyanamid Co., Agric. Chem. Div., New York, N. Y. [1947].

FACTORS INFLUENCING SPRAY INJURY BY GLYOXALIDINE DERIVATIVES TO POTTED APPLE TREES[1]

S. E. A. McCallan

The fungicidal properties of glyoxalidine derivatives were first reported by Wellman and McCallan (15) in 1945. Since then certain of the derivatives have been rather widely tested as foliage sprays, especially for apple scab and cherry leaf spot, where in general they have given good control (1, 2, 3, 4, 5, 6, 7, 14). Failure to control apple scab in 1946 has been ascribed by Harry, McNamee, and Wellman (10) to hydrolysis of the glyoxalidine derivatives. However, at various times there have been reports (1, 2, 3, 4, 5, 6, 7) of injury to both apple foliage and fruit. Accordingly a preliminary study has been made under laboratory and greenhouse conditions to elucidate, if possible, the factors involved.

MATERIALS AND METHODS

The apple trees (*Pyrus malus* L.) were the variety McIntosh on dwarf Malling IX stock grown in six-inch pots. A supply of potted plants was maintained in cold storage at 0.5° F. and removed to room temperatures when required for use. The trees were severely pruned back and used for the injury studies when the shoots were about six inches or longer and held six or more leaves. During the early summer when growth was more active it was possible to use the same plants again as continued elongation had produced more leaves. Duplicate or triplicate potted plants were used for a given variable in all cases and a total of over 400 plants was observed.

The study was based primarily on a 50 per cent solution of acetates of "mixed glyoxalidines" in isopropanol, that is, the 1949 341C formulation. The "mixed glyoxalidines" consist of approximately 65 per cent 2-heptadecylglyoxalidine, 32 per cent 2-pentadecylglyoxalidine, and less than 3 per cent 2-heptadecenylglyoxalidine. A quart of 341C formulation is equivalent to three-quarter pound of glyoxalidine derivatives.

Tests were also made under the more important conditions on the 1949 341SC formulation. This is a modification of the C formulation in that the glyoxalidine was prepared from 98 per cent pure stearic acid and consists essentially of pure 2-heptadecylglyoxalidine in isopropanol; practically no 2-heptadecenylglyoxalidine is present. The SC formulation, however, is less concentrated, one quart containing only the equivalent of one-half pound of glyoxalidine. In addition some limited observations were made

[1] These results were reported before the American Phytopathological Society, New York, N. Y., December 1949 (12).

Copyright, 1950, by Boyce Thompson Institute for Plant Research, Inc.

on the 1949 B formulation which consists of 30 per cent "mixed glyoxalidines" and 3.3 per cent of 1-hydroxyethyl-2-heptadecylglyoxalidine dispersed on kaolin, and on a wet ball milled sample. The fungicidal applications were made under the standard conditions of the spray laboratory by means of a touch-up paint gun at 40 lb. pressure (11, 13).

FACTORS STUDIED

The various factors studied were as follows:

Concentration. The standard field concentration for 341C, namely, 0.25 per cent or one quart per 100 gallons of spray, was applied. However, since the variety McIntosh is not particularly sensitive to injury from 341, a four-fold or 1.0 per cent concentration was also applied. In the case of 341SC which contains only two-thirds as much active ingredient per volume of SC the necessary correction was made to give concentrations equivalent to those employed for 341C. The standard and four-fold concentrations were used in all tests. Also, when appropriate, unsprayed controls were carried.

Lime. The presence and absence of lime was studied in most of the tests. The standard ratio of calcium hydroxide was used, namely 5 g.+10 ml. 341C formulation to make 1,000 ml. of a 1 per cent spray.

Temperature. Temperatures of approximately 35°, 50°, 70°, 80°, 90°, and 100° F. were obtained in controlled chambers to which the sprayed plants usually were exposed for 20 to 24 hours. In some cases, the sprayed plants were exposed to freezing temperatures of from 28° to 30° F. for one-half hour before exposure to various of the above temperatures.

Humidity. Saturated atmospheres were also studied at temperatures of 50°, 70°, 80°, and 90° F., in contrast to dry air (25 to 50 per cent relative humidity) for all the temperatures available.

Soil moisture. The effect of sprays on plants grown in normal watered pots was compared to plants not watered for two days prior to spraying in which the soil had dried out and the plants showed obvious signs of wilting.

Duration of exposure. Plants were exposed for short periods of four to six hours to certain of the factors studied in contrast to the longer and usual exposures of 20 to 24 hours.

Duration of time between spraying and exposure. Normally all plants were exposed to the specified condition immediately after spraying. In one test, the plants were held out-of-doors for periods of one, three, seven, and fourteen days before exposure.

RESULTS

It was found that injury could be induced on the young foliage which was apparently similar to that reported in the field as early season injury from 341C. This is typified by a bronzing of the lower surface of the leaf,

TABLE I

Summary of Injury from Glyoxalidine Derivatives Expressed in Arbitrary Units

	Factor	Average response*
A**	Control	0
	Standard concentration (0.25%)	1.2
	4× standard concentration (1.00%)	2.5
B	Lime	1.6
	No lime	1.7
C	35° F.	0.3
	50° F.	0.5
	70° F.	1.0
	80° F.	1.8
	90° F.	2.2
	100° F.	2.0
D	Humid air	2.2
	Dry air	1.5
E	Moist soil	2.0
	Dry soil	1.8
F	4–6 hrs. at 90° F.	2.2
	20–24 hrs. at 90° F.	2.4
G	Time between spraying and exposure at 90°	
	0	3.3
	1 day	2.5
	3 days	3.0
	7 days	1.5
	14 days	0.8
H	341C formulation	1.9
	341SC formulation, no lime	0.9
	341SC lime	0.2

* Scale, 0 no injury to 5 for severe injury.
** Formulation 341C used in Sections A to G.

of varying extent. In the mildest cases, a slight bronzing was barely noticeable on a small area, while in the more serious cases the whole lower leaf was involved and speckled necrotic areas appeared through on the upper surface. This response was usually confined to the younger but more or less fully developed leaves situated several leaves below the leaf bud. Less typical and common symptoms were a necrosis of the margins and center portions of the youngest leaves just emerging from the bud, that is, the very crown of the plant. As the plant aged, the bronzing appearance of the lower leaves which were young when sprayed, took on more of a whitened effect. The extent of bronzing was characterized as none, very slight, slight, moderate, severe, and very severe. In order to simplify tabulation, averaging, etc., these degrees of injury were given numeral ratings respectively of zero, one, two, three, four, and five.

The results from the various factors studied are summarized in Table

TABLE II

COMPARISON OF CONCENTRATION, LIME, TEMPERATURE, AND FORMULATION ON INJURY FROM GLYOXALIDINE DERIVATIVES EXPRESSED IN ARBITRARY UNITS*

	90° F.		
	341C formulation	341SC formulation	
		No lime	Lime
Control	0	0	0
Standard concentration	1.1	0.6	0.4
4 × concentration	3.6	2.8	0.8
	70° F.		
Control	0	0	0
Standard concentration	0.8	0	0
4 × concentration	2.0	2.0	0

* Scale, 0 no injury to 5 for severe injury.

I. The numeral values represent the average injury response for the factor specified. The average, however, is obtained from a number of different conditions. Thus within a section, for example B, lime and no lime, direct comparisons can be made since the values are the result of a number of paired comparisons under different conditions. However, direct comparisons between sections, for example A versus B, should not be made as the conditions may be somewhat different.

It will be seen in Section A that no injury was observed on the checks. However, injury increases with increasing concentration and more than doubles in the change from standard to four-fold concentration. The presence or absence of lime was studied in almost all experiments and, in general, no overall difference could be noted as seen in Section B. Henceforth all tabular references to 341C, for example Section H and Table II, are the average results of lime and no lime tests.

The effect of temperature is summarized in Section C. Injury increases rapidly with higher temperatures and is marked at 90° F. The apparent injury at 100° F. is less than at 90° F. This, however, is due to the fact that the conditions are not comparable at both temperatures as will be explained below. Experiments at below freezing temperatures, not shown, resulted in mild freezing injury, but not in characteristic 341 injury. It is interesting to note that the reports of injury in 1949 from early season spray (7) came largely from the relatively southern areas comprising North Carolina, New Jersey and the Shenandoah, Cumberland, and Hudson valleys where the early spring had been unusually warm. In northern New England and in Canada the glyoxalidine sprays performed satisfactorily without reports of injury. Under laboratory conditions the older leaves appeared resistant to spray injury; this perhaps correlates with the field observation that injury in the above areas was not noted after petal fall.

An atmosphere saturated with moisture is much more conducive to injury than a relatively dry atmosphere as shown in Section D of Table I. The results at 100° F. alone are based only on a dry atmosphere, whereas those at 90° F. to 50° F. include both dry and saturated atmospheres. The plants growing in a dry soil and in a wilted or near wilted condition, Section E, appear more resistant to injury. There is, however, relatively little information on this factor.

Injury apparently occurs soon after exposure to unfavorable conditions, that is, 90° F., and is not appreciably increased by prolonging the time from a few hours to 20 or 24 hours, as is shown in Section F of Table I. Sprayed plants held out-of-doors for periods up to three days before exposure to high temperature and high humidity showed no diminution in injury, Section G. The injury declined somewhat at seven days and very much at fourteen days, though in part this may have been due to rain which fell during the period.

In the last section, H, of Table I, there is a direct comparison of 341C and SC formulations. 341SC without lime is appreciably less injurious than C, and when lime is added the decrease in injuriousness becomes very pronounced.

Limited results not given in Table I indicate that 341B formulation shows about the same order of toxicity as 341SC and that a wet ball milled sample of 341 is more injurious than these but less than the 341C formulation.

The detailed results are given in Table II of a comparison of 341C and 341SC with and without lime at the different concentration levels and at 70° F. and 90° F. both at 100 per cent relative humidity. It will be seen that at 70° F. even the four-fold concentration of the 341SC formulation with lime causes no injury, while at 90° F. the standard concentration produced relatively little injury. The data here show that 341SC with lime is less than one-quarter as injurious as 341C. Other laboratory studies, and field data by Chandler and Thurston (8), indicate that the non-injuriousness of the 341SC formulation is due to its content of essentially pure 2-heptadecylglyoxalidine, while the injuriousness of the 341C formulation is due primarily to the unsaturated 2-heptadecenylglyoxalidine and secondarily to the pentadecyl derivative.

Scab control. Greenhouse studies with apple scab on the dwarf McIntosh plants have been performed using the spray laboratory equipment (11, 13) and following the general procedures of Hamilton and Mack (9) for handling the trees and scab organism. The results of three tests with three replicate plants at each of three concentrations gave a mean ED95 value of 0.012 per cent (active ingredient) for 341C and 0.018 per cent for 341SC; these differences are not significant. Field tests in the Institute dwarf McIntosh orchard, using five replicate trees per concentration and comparing 341C and 341SC at one-half and two pounds (active ingredient) per 100 gallons gave identical control of foliage scab.

SUMMARY

Spray injury from glyoxalidine derivatives was induced on the young leaves of potted apple trees under laboratory conditions. This bronzing of the lower surface resembled that reported from early season sprays with 341C formulation.

The most important factors are high concentration, high temperatures of 90° F. or above, and high humidity.

The 341SC formulation containing essentially pure 2-heptadecylglyoxalidine, plus lime, is less than one-quarter as injurious as the 341C formulation containing a mixture of different glyoxalidines.

LITERATURE CITED

1. AMERICAN PHYTOPATHOLOGICAL SOCIETY. POTOMAC DIVISION. FUNGICIDE COMMITTEE. Progress in testing the relative effectiveness of new organic fungicides in the control of plant diseases: A summation of nation-wide data in 1945. [Mimeographed] 21 pp. 1946.
2. [———] 1946 Fungicide tests: A summation of nation-wide results with newer fungicides. [Mimeographed] 29 pp. 1947.
3. [———] Report of the special committee on the coordination of field tests with new fungicidal sprays and dusts, with reference to results obtained in 1947. Plant Dis. Reptr. Suppl. 174: 41–86. 1948.
4. ——— Fungicide Sub-committee on "Summation of the Performance of Newer Fungicides." 1947 Fungicide tests: A summation of nation-wide results with newer fungicides. Plant Dis. Reptr. Suppl. 176: 95–142. 1948.
5. ——— Nation-wide results with fungicides in 1948. Fourth annual report. Plant Dis. Reptr. Suppl. 181: 17–87. 1949.
6. [———] Second annual report of the special committee on the coordination of field tests with new fungicidal sprays and dusts, with reference to the results obtained in 1948. Plant Dis. Reptr. Suppl. 183: 112–177. 1949.
7. ——— [Special Committee on] Coordination of Field Tests with New Fungicidal Sprays and Dusts. Report of the section on fungicide tests for the control of apple diseases, 1949. J. M. Hamilton [chairman]. [Mimeographed] 13 pp. [1949].
8. CHANDLER, W. A., and H. W. THURSTON, JR. The constituents of "341" as apple scab fungicides. Phytopath. **39**: 4. 1949.
9. HAMILTON, J. M., and G. L. MACK. New and improved methods for study of fungicides. Farm Research. [Geneva, N. Y.] **8**(1): 10, 13. 1942.
10. HARRY, JOHN B., R. W. MCNAMEE, and R. H. WELLMAN. Heptadecylgloxalidine (341) and its hydrolysis product as fungicides. Phytopath. **37**: 9. 1947.
11. MCCALLAN, S. E. A. Some improvements in equipment for evaluating fungicides by the foliage disease method. Contrib. Boyce Thompson Inst. **15**: 71–75. 1948.
12. ——— Factors influencing glyoxalidine spray injury to potted apple trees. Phytopath. **40**: 19. 1950.
13. MCCALLAN, S. E. A., and R. H. WELLMAN. A greenhouse method of evaluating fungicides by means of tomato foliage diseases. Contrib. Boyce Thompson Inst. **13**: 93–134. 1943.
14. THURSTON, H. W., JR., JOHN B. HARRY, F. H. LEWIS, A. B. GROVES, and C. F. TAYLOR. Glyoxalidine derivatives as foliage fungicides. II. Field studies. Contrib. Boyce Thompson Inst. **14**: 161–171. 1946.
15. WELLMAN, R. H., and S. E. A. MCCALLAN. Glyoxalidine derivatives as foliage fungicides. I. Laboratory studies. Contrib. Boyce Thompson Inst. **14**: 151–160. 1946.

FACTORS INFLUENCING DEPOSITION IN THE VACUUM BELL JAR DUSTER

S. E. A. McCallan

A vacuum bell jar duster has been described by Farrar, O'Kane, and Smith (1) for use in insecticide tests. Recently Richards and Murphy (5) have used one for tests with fungicides, working specifically with bean rust. Since this apparatus appears likely to become of more general use in the laboratory evaluation of insecticides and fungicides, a study has been made of various factors influencing deposition.

APPARATUS

A vacuum bell jar duster was built following the general specifications of Farrar *et al.;* however, several modifications were introduced to meet local conditions, or because they appeared to be improvements.

The bell jar measured 20.5 cm. inside diameter and 35 cm. inside height, with a neck 5.0 cm. long and 3.6 cm. inside diameter. Since the apparatus was employed on a bench at the side of a hood in the spray room (2) the method of mounting and exhausting was changed. The bell jar was mounted on a smooth slab of alberene stone 26 cm. square and 3 cm. thick. A sheet of hard rubber was glued to the upper surface of the stone to serve as a gasket and to protect the surface of the stone. In addition a ring-shaped gasket was cut slightly larger than the base of the bell jar. The two gaskets provided an excellent seal between the bell jar and stone. The potted plants to be dusted were placed directly on the center of the rubber-covered stone. The bell jar was wrapped with fly screen wire for protection in case of breakage.

The metal dusting unit or loading device illustrated in Figure 1 was constructed of a stainless steel cap 6.5 cm. in diameter and 1.2 cm. thick. Through this cap was inserted a stainless steel tube of 1.2 cm. inside diameter, which projected 1.2 cm. above the cap and 1.8 cm. below. Two brass supporting rods approximately 0.3 cm. in diameter were screwed into the lower surface of the cap either side of the tube. These rods were 5.7 cm. long and at the lower end were brazed to a brass ring. This ring replaces the concave disc of the original (1) and together with its accompanying watch glass is considered the major improvement in the vacuum dusting apparatus. The ring is 3.1 cm. in diameter and approximately 2.5 mm. thick. At about 3 mm. from the outside edge of the ring it is bored out to hold a standard 25 mm. watch glass. The upper inside diameter of the ring is thus slightly more than 2.5 cm. and the lower about 1.7 cm.; the

Copyright, 1950, by Boyce Thompson Institute for Plant Research, Inc.

intervening lip supports the watch glass, the upper rim of which is flush with the upper surface of the ring. The lower surface of the steel cap was fitted with a hard rubber gasket to provide a seal against the ground glass top of the bell jar.

The use of the watch glasses simplified the loading apparatus, since accidents are likely to occur when transferring a small amount of dust to the loading pan under the relatively rough conditions of a spray laboratory. Since the watch glasses are not of exactly the same diameter, it will be necessary to discard a small percentage which are too large or too small in order to get a good fit in the ring.

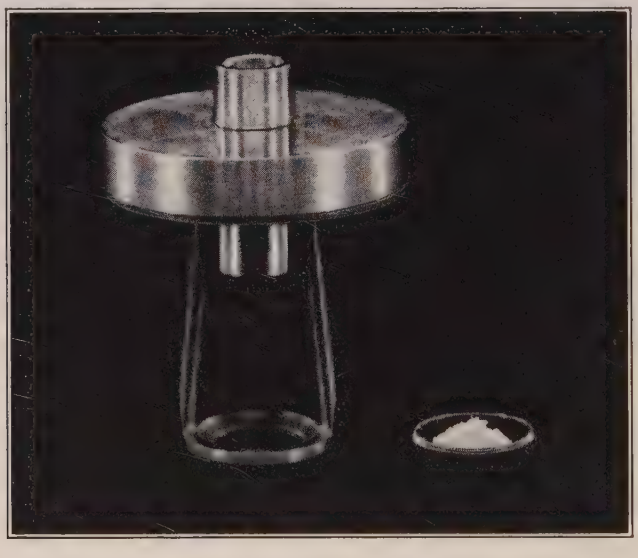


FIGURE 1. Loading unit with watch glass holding dust at side. Watch glass is placed in beveled ring when ready to use.

As now employed the amounts of dust are weighed out in the laboratory and immediately transferred to the 25 mm. watch glasses. The individual watch glasses are placed on a tray equipped with a false bottom 30×43 cm. in which are bored a series of 70 holes (7×10) 2 cm. in diameter, as illustrated in Figure 2. Each hole will carry a watch glass in a fairly stable position and in addition the tray is provided with a cover sufficiently high to clear the piles of dust on the watch glasses. The identification of the dust samples is recorded by position on the tray.

A general laboratory vacuum line was available and the pressure was regulated by a valve and read on a mercury manometer. It was found desirable to insert a trap between the manometer and vacuum line, which consisted of a 1 liter vacuum flask filled with glass wool. About 100 cm.

of pressure tubing is needed between the trap and side entrance of bell jar where it is attached in order to provide flexibility for moving and exhausting the bell jar.

After dusting, the bell jar is placed over a box attached to the hood. The box is 60 cm. long (the width of the hood window), 30 cm. wide, and 10 cm. high and is closed except for one side and a hole in the top. The open side fits next to the hood, the window of which is closed upon the box. The bell jar is placed directly over the hole (20.5 cm. diameter) where it is rapidly exhausted (Fig. 3 B).

The plant to be dusted is placed under the bell jar. The loading unit is held on a ring support and the watch glass containing the dust samples is

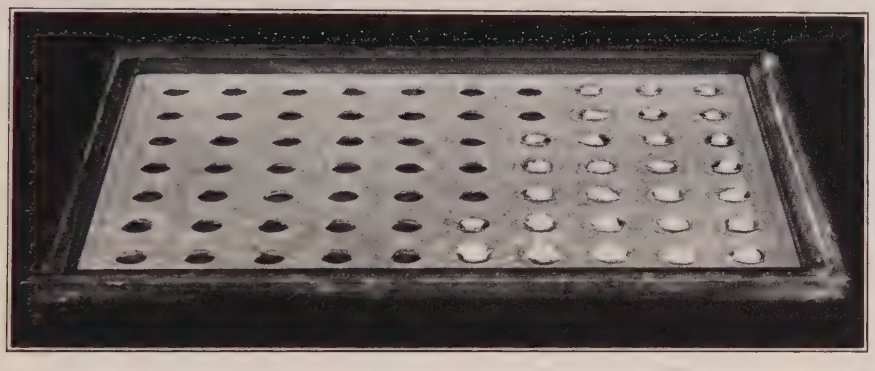


Figure 2. Tray to hold watch glasses of dust.

inserted on the ring. The unit is then placed in position in the neck of the bell jar and the steel tube closed by placing a rubber stopper on top (Fig. 3 A). The bell jar is evacuated to the desired pressure and the stopper quickly removed. The bell jar is raised, the dusted plant removed, and the jar placed on the exhaust box. If necessary the bell jar is wiped out with a clean cloth. It is then placed over a fresh plant and the process repeated.

METHODS

These studies on deposition were confined to bean plants (*Phaseolus vulgaris* L.) of the varieties Pinto and Tendergreen grown in 3-inch pots and before use thinned to single or duplicate plants per pot. The plants were used when the pair of primary leaves was from one-half to fully expanded and before there was an appreciable growth of other leaves. In order to have a means of measuring the amounts of dust in the leaf deposit, 10 per cent of brilliant blue dye powder[1] by weight was thoroughly incorporated in the dust. A microscopic examination showed that the dye

[1] Brilliant Blue F.C.F. Coal Tar Dye. Eimer & Amend, New York, N. Y.

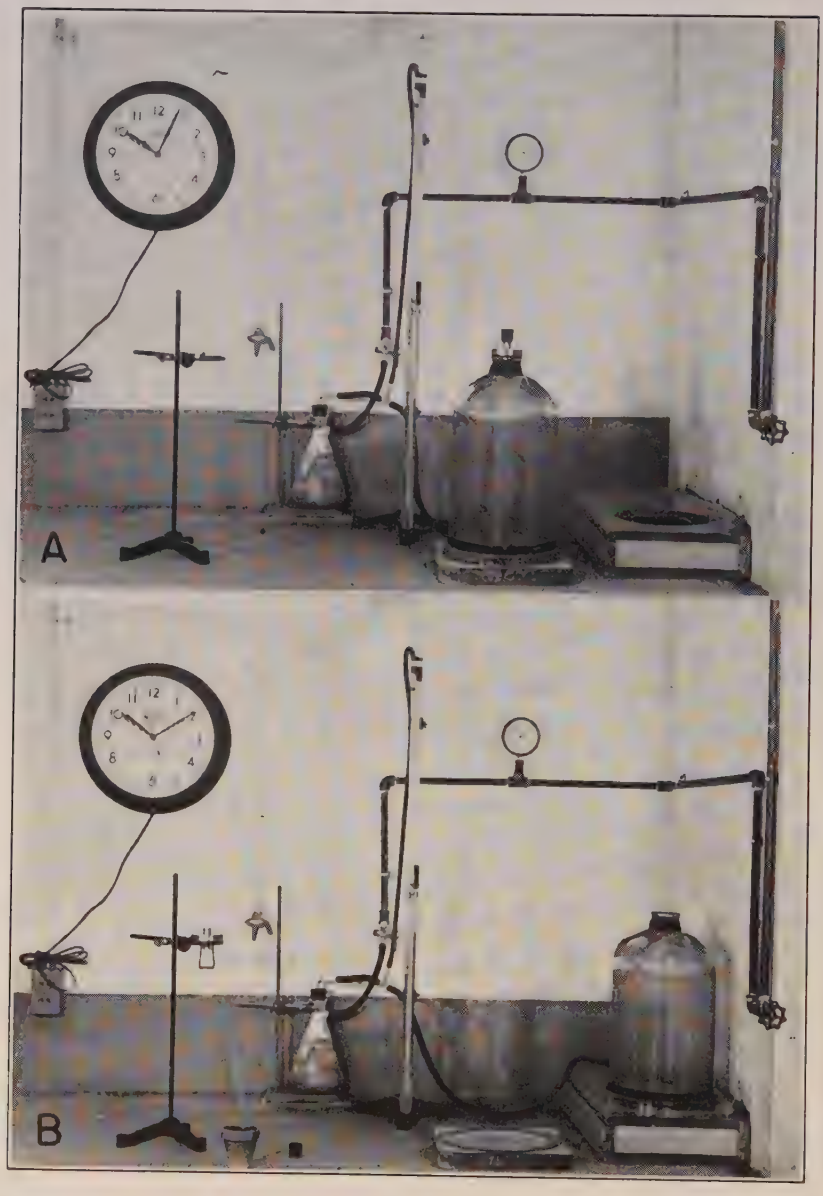


FIGURE 3. A. Vacuum bell jar assembly with potted plant under bell jar, loading unit in position in neck of jar, rubber stopper on top of loading device closing system which has been evacuated to desired pressure. Removing stopper will discharge dust. Note manometer and suction flask trap. B. Bell jar being exhausted on box inserted at side of hood. Note that loading unit has been returned to extension ring on tripod ready to be loaded with another watch glass of dust.

powder was uniformly distributed throughout the dust. The dust consisted of Pyrax ABB diluent unless otherwise stated, and other carriers.

After deposition the dust was recovered from each leaf by washing the surface carefully with 10 ml. of distilled water delivered from a pipette. Since the blue dye is water soluble the amount of dust recovered could be determined readily in a colorimeter. The area of each leaf was also determined by means of a planimeter and the amount of dust deposited was expressed as milligrams per square decimeter of leaf surface.

EXPERIMENTAL RESULTS

The factors deemed of importance in deposition and studied separately were: area of leaf surface; weight of dust sample; pressure; height of plant, *i.e.*, distance between plant and watch glass; time of deposition; number of plants in duster; upper and lower leaf surfaces; types of dust diluents; and variation between paired leaves, paired plants, and replicate tests.

Area of leaf surface. Deposition on leaves of different areas (total for both surfaces) was studied for sample weights of 25, 50, 100, and 200 mg. A constant reduced pressure of 240 mm. mercury within the bell jar and 5 seconds time was maintained here, as in other cases, where these two factors were not under test. A reduced pressure of 240 mm. Hg results in a difference of approximately 10 lb. per sq. inch between the inside of the bell jar and the outside atmosphere. The scatter diagrams obtained for the above samples are shown in Figure 4 A. Highly significant linear regressions (7) were obtained for dust deposition on leaf area; thus it may be concluded that deposition is directly dependent on leaf area. Hereafter the difference in leaf area was corrected for, by expressing the results for a constant area of 1 sq. decimeter.

Weight of sample. The most convenient size of sample for this apparatus is 50 or 100 mg., but deposition over the range 25 to 300 mg. was investigated. The results shown in Figure 4 A calculated on the basis of unit leaf area are presented in Figure 4 B. It will be seen that deposition is directly proportional to size of sample. This has been confirmed in many subsequent tests.

Pressure. The bell jar was evacuated to reduced pressures of 440, 340, 240, 140, and 40 mm. of mercury and the resulting deposition determined. Results based on two different weights of sample (50 and 100 mg.) and two sets of paired plants, *i.e.*, 16 separate leaves and determinations per pressure, are give in Figure 5. The relation here appears to be approximately linear with a small increase in deposition (mean of both leaf surfaces) of 0.16 mg. per sq. dm. per 100 mm. Hg. decrease in pressure within the bell jar. The linear regression as calculated falls just short of significance (7).

Distance between dust sample and leaf. Potted bean plants were placed

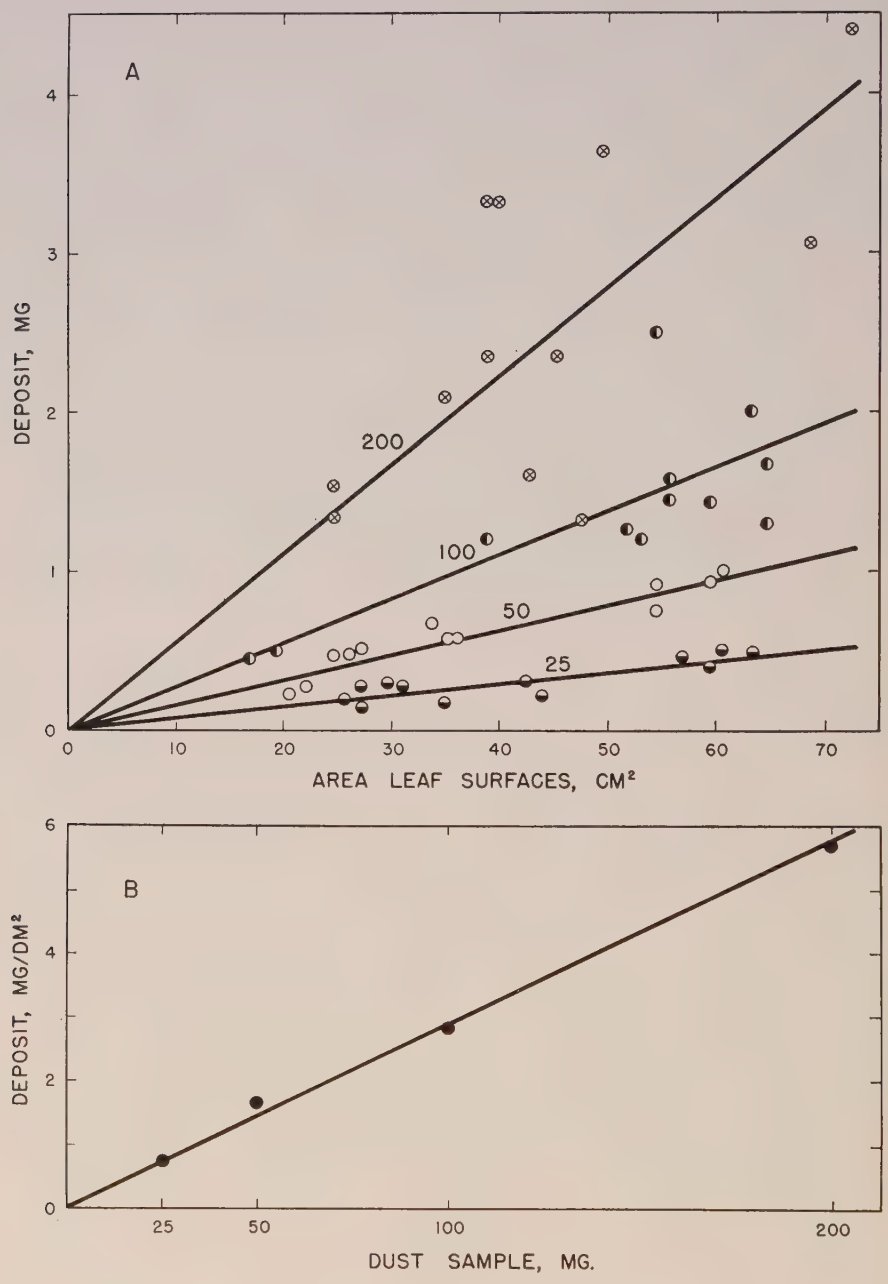


FIGURE 4. A. Dust deposits on individual leaves for 25, 50, 100, and 200 mg. samples. B. Above data calculated to show linear relation between size of sample and amount of deposition per unit area.

under the bell jar, (a) on the alberene stone, (b) 5 cm. above it, and (c) 10 cm. above. Deposition studies were made as in the pressure series on 16 separate leaves per height. An analysis of variance (7) failed to show any significant difference in deposition due to height. It is thus apparent that differences in heights of plants, at least within the range studied, are without appreciable effect on the final deposition.

Number of plants. A comparison was made of the total deposit on single plants as compared to two plants in a pot exposed at the same time. Data

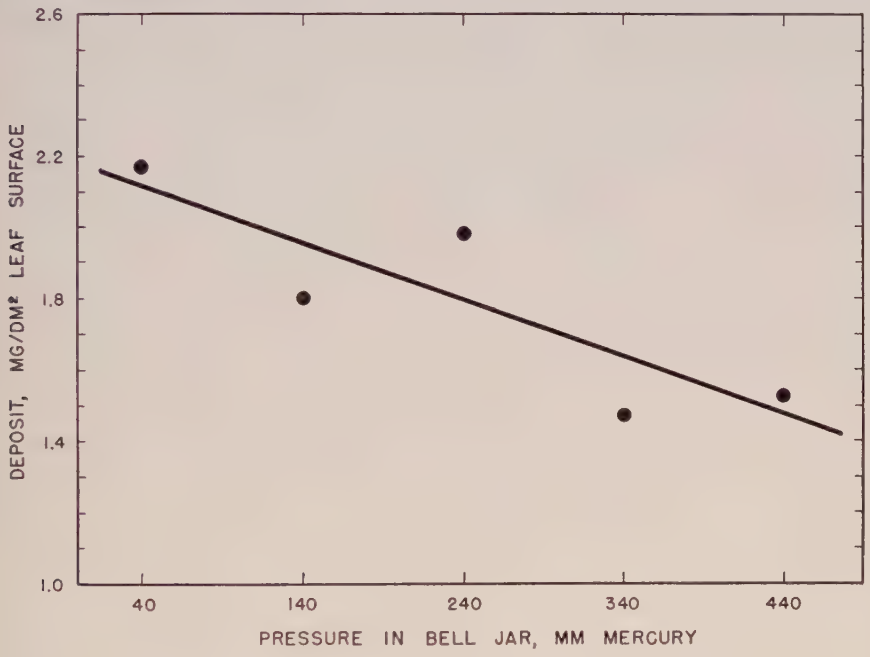


Figure 5. Relation of deposition to pressure within bell jar.

based on two single plant exposures and two double plant exposures for 25, 50, 100, and 200 mg. samples showed a consistent difference of 12 per cent heavier deposit for the single plants. This difference is presumably due to the interference of one leaf on another in the case of the two plants. No significant difference in variation of amount deposited per plant could be shown between duplicate plants in the same pot dusted at the same time and single plants dusted in succession.

Time of deposition. In studying deposition times it was essential to remove the rubber stopper very sharply and to lift the bell jar from the plant in a similar and precise manner. Times of 1, 2, 5, 10, 20, 40, and 80 seconds between removing stopper and plant from bell jar were studied. Dust samples of 50, 100, and 200 mg. and replicated single plants were

used. The whole series of tests was repeated a second day. In all, 24 separate leaf analyses were made per time exposure. The results of one series are illustrated in Figure 6.

Upper and lower leaf surfaces. The data shown in Figure 6 were obtained separately for both leaf surfaces. It will be seen that at the shortest times little if any difference between the two surfaces is evident. However, as time elapses the deposit continues to build up on the upper surface. These data and others indicate that after about 5 seconds there is little further

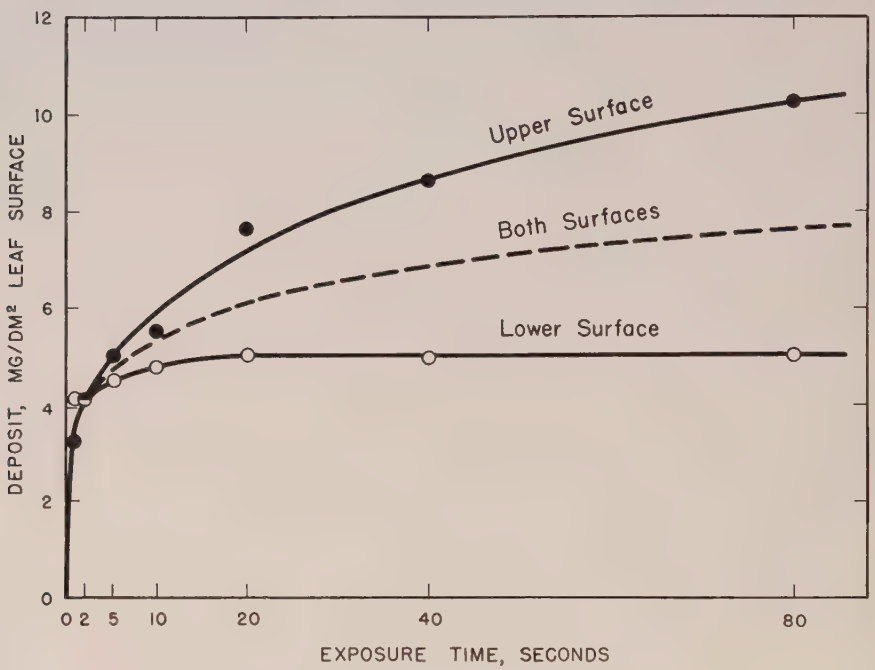


FIGURE 6. Relation of deposition to exposure time for upper and lower leaf surfaces.

deposition on the lower surface and that on the upper surface is due to gravity. Accordingly a time lapse of 5 seconds has been adopted as standard. It should be noted that since deposition for the upper surface is continuing the 5 seconds' exposure should be carefully determined when making comparative evaluations of dusts.

Diluents. A series of 10 representative dust diluents listed in Table I was selected on the basis of the classification of Watkins and Norton (9) and was compared with Pyrax ABB used above. Mean results of a series of tests with the different diluents based on two single plants and three weights of samples are given in the last column of Table I. There is considerable difference among the diluents in the amount deposited. The phy-

TABLE I

Dust Diluents Employed, with Mean Deposition in Mg. per Sq. Dm. for Samples of 50, 100 and 200 Mg.

Type	Trade identification	Manufacturer	Deposition
Wood flour	G28T8N20	Waterside Milling Co., Tacoma, Washington	2.04
Silicon dioxide	Celite 209	Johns Manville Sales Corp., New York, N. Y.	3.06
Calcium hydroxide	Hydrated lime	Mallinckrodt Chem. Works, St. Louis, Mo.	2.78
Calcium carbonate	Calcium carbonate	Mallinckrodt Chem. Works, St. Louis, Mo.	2.08
Calcium sulfate	Gypsum		2.24
Talc	Talc, grade #41	Eastern Magnesium Talc Co. Inc., Burlington, Vermont	2.29
Pyrophyllite	Pyrax ABB	R. T. Vanderbilt Co. Inc., New York, N. Y.	1.71
Montmorillonite	Wyobond Wyoming bentonite 200 mesh	Wyodak Chem. Co., Cleveland, Ohio	2.17
Kaolinite	Cherokee clay	R. T. Vanderbilt Co. Inc., New York, N. Y.	1.39
Attapulgite	Attaclay	Attapulgus Clay Co., Philadelphia, Penn.	1.99

sical state of suitable dust diluents used in the vacuum bell jar duster calls for certain physical properties such as a fluffy free-flowing dust, medium volume for a given weight, and absence of excessive "smoking" after removing the bell jar. Five of the dusts which met these criteria were reexamined and the relative data on deposition were confirmed. An analysis of variance indicated that the samples of talc, bentonite, Pyrax ABB and Attaclay tested did not differ significantly in amount deposited on leaves, as shown in Table II. However, the Cherokee clay deposited only about two-thirds as much. All five diluents, however, would be considered suitable for use with this apparatus.

TABLE II

Deposition of Dust Diluents. Mean Mg. per Sq. Dm. for Two Plants and Two Tests

Diluent	Sample, wt. in mg. 50	100	200	Mean
Talc	0.98	2.06	4.17	2.40
Bentonite	1.14	2.03	3.80	2.32
Pyrax	0.85	1.94	3.28	2.02
Attaclay	1.10	1.99	2.91	2.00
Cherokee clay	0.69	1.22	2.05	1.32
LSD 19:1				0.41

Sources of variation. In addition to the controllable sources of variation discussed above there are various factors which in general cannot be con-

trolled and which introduce errors or variation into replicate determinations. These variations appear to be due to three major sources: the variation between deposition on opposite leaves of the same plant, between different plants dusted in the same experiment, and between experiments performed at different times, that is, day to day variation (3).

Representative data for deposition have been collected and analyzed for 50, 100, and 200 mg. samples of Pyrax ABB dust; 240 mm. Hg. pressure; 5 seconds' deposition time; on opposite leaves; single plants replicated twice; and the entire experiment composed of two plants or four leaves per sample repeated on different days over a period of time. The combined analyses gave the following results:

	DF	*SS*	*MS*
Between experiments	17	56.7096	3.3359
Between plants of same experiment	20	17.4936	0.8747
Between leaves of same plant	40	14.7886	0.3697

It is interesting to compare the relative magnitude of these three sources of variation. However, following the reasoning of Tippett (8, p. 92), Snedecor (6), and others on the components of variance, it may be concluded that the mean square for "between plants" is made up of two variances, that attributable to different plants plus that attributable to different leaves of the same plant. Likewise the "between experiments" error is composed of three separate estimates of error, that due to different experiments, to different plants, and to different leaves. By employing the procedures described above the following "corrected" variances were obtained:

	V	*Coefficient of variation*
Between experiments	0.6153	25.5
Between plants	0.2525	16.4
Between leaves	0.3697	19.8

It will be seen that the magnitude of error due to different leaves or to different plants is about the same, giving respectively coefficients of variation of 19.8 per cent and 16.4 per cent. The errors due to different experiments or day to day variation, as is generally to be expected, are somewhat greater with a coefficient of variation of 25.5 per cent. These errors are considerably in excess of those obtainable for the very precise settling tower method of applying sprays (4) but are probably reasonable for the application of dusts.

SUMMARY

1. A vacuum bell jar duster was constructed following the general principles of Farrar *et al.*, but modified to carry the samples of dust in movable 25 mm. watch glasses. The apparatus was also designed for use with a hood.

2. Deposition was studied on bean plants using dusts mixed with dyes.

The water soluble dye was recovered from the deposited dust and measured in a colorimeter and the amount of dust deposit calculated.

3. Deposition is directly dependent on weight of sample and area of leaf. The deposit increases slightly with decrease in pressure within the bell jar; 240 mm. Hg was adopted as standard. Height of plant *i.e.*, distance from dust sample, was without effect on amount of deposit.

4. The deposit on the lower surface of the leaf does not continue to build up much after 5 seconds from time of discharging dust. However, deposition on the upper surface may continue for a minute or more. Five seconds was taken as standard time.

5. Ten different representative dust diluents were studied and found to differ considerably in desirable dusting qualities and amount deposited. Satisfactory diluents were samples of Eastern Magnesium talc, Pyrax ABB, Wyobond Wyoming bentonite, Cherokee clay, and Attaclay.

6. The primary source of error is day to day variation in experiments giving a coefficient of variation of 25 per cent while the error of different plants and different leaves from the same plant contribute respectively an additional 16 and 20 per cent variation.

LITERATURE CITED

1. Farrar, M. D., W. C. O'Kane, and Howard W. Smith. Vacuum dusting of insects and plants. Jour. Econ. Ent. **41**: 647–648. 1948.
2. McCallan, S. E. A. Some improvements in equipment for evaluating fungicides by the foliage disease method. Contrib. Boyce Thompson Inst. **15**: 71–75. 1948.
3. McCallan, S. E. A., R. H. Wellman, and Frank Wilcoxon. An analysis of factors causing variation in spore germination tests of fungicides. III. Slope of toxicity curves, replicate tests, and fungi. Contrib. Boyce Thompson Inst. **12**: 49–77. 1941.
4. McCallan, S. E. A., and Frank Wilcoxon. An analysis of factors causing variation in spore germination tests of fungicides. II. Methods of spraying. Contrib. Boyce Thompson Inst. **11**: 309–324. 1940.
5. Richards, M. C., and Douglas Murphy. The vacuum duster for applying fungicides and inoculum to plants. Phytopath. **39**: 20. 1949.
6. Snedecor, George W. Biological variation vs. errors in measurement. Science **80**: 246–247. 1934.
7. ——— Statistical methods. 4th ed. 485 pp. Iowa State College Press, Ames, Iowa. 1946.
8. Tippett, L. H. C. The methods of statistics. 222 pp. Williams & Norgate Ltd., London. 1931.
9. Watkins, Thomas C., and L. B. Norton. Properties and commercial sources of insecticide dust diluents and carriers. Mimeographed 259 pp. Agric. Insect. & Fungic. Assoc., New York, N. Y. 1947.

LYGUS BUGS IN RELATION TO SEED PRODUCTION AND OCCURRENCE OF EMBRYOLESS SEEDS IN VARIOUS UMBELLIFEROUS SPECIES

Florence Flemion and June Olson

Many of the freshly-harvested seeds of the Umbelliferae which appear well-filled and normal fail to germinate because they do not have embryos (4). The embryoless condition is apparently caused by the tarnished plant bug (*Lygus oblineatus* Say) since it has been shown (3) that many such seeds are produced on plants of dill and carrot which have been exposed to feeding at various stages after the blossoming period. Control plants protected from *Lygus* bugs are essentially free of embryoless seeds.

The data presented in the present paper show that feeding by *Lygus* bugs also results in embryoless seeds in other umbelliferous species such as celery (*Apium graveolens* L.), coriander (*Coriandrum sativum* L.), fennel (*Foeniculum dulce* Mill.), goutweed (*Aegopodium podagraria* L.), parsley (*Petroselinum hortense* Hoff.), and parsnip (*Pastinaca sativa* L.). Seed yield of dill and parsnip was also greatly reduced when *Lygus* bugs were permitted to feed during and immediately following the flowering period. Other insects tested such as stinkbug (Pentatomidae sp.), spotted cucumber beetle (*Diabrotica duodecimpunctata* F.), and smartweed flea beetle (*Systena hudsonias* Forst.) caused some injury but did not produce embryoless seed.

MATERIALS AND METHODS

Plants of coriander, dill, and fennel were grown in the greenhouse from seed while plants of both wild and cultivated carrot, celery, parsley, and parsnip were grown out-of-doors, the roots having over-wintered outside in soil. Plants of goutweed, an annual, were collected in the wild. All of the experiments described below were conducted in 1949 with plants of these various umbelliferous species which had been transplanted to eight-inch clay pots containing fertile soil.

Prior to flowering the plants were sprayed with 0.125 per cent emulsion of 40 per cent TEPP (tetraethyl pyrophosphate) (3, p. 306) and then placed in insect-free cages. Houseflies (*Musca domestica* L.) (3, p. 300) were kept continuously in all of the cages during the flowering period to serve as pollinators. The various other insects added to the cages (for technique see 3, p. 300–301) were all collected in Yonkers, New York, except three large shipments of *Lygus* bugs (tarnished plant bug) obtained in early June—one collected by Harriet Poole at Freehold, New Jersey, and two through the courtesy of the Department of Entomology at Rutgers University collected in the vicinity of New Brunswick, New

Coypright, 1950, by Boyce Thompson Institute for Plant Research, Inc.

Jersey. The insects were identified by Dr. Albert Hartzell and Dr. Harry L. Haynes of this Institute.

The supply of insects in each cage was replenished periodically so that several could be detected at all times without much difficulty. Although the number of insects was not rigidly controlled, at least 30 adult *Lygus* bugs were placed in each cage ($2' \times 3' \times 5\frac{1}{2}'$ high, for illustration see 3, p. 301) at the beginning of the experiment and more were added when the number seemed to diminish. In one experiment, *Lygus* were detected in the non-infested cage so they were eliminated by spraying with TEPP and thereafter all control plants and cages were similarly sprayed at periodic intervals. Nicotine (0.1 per cent) was used for the control of aphids on all the plants whether caged with or without *Lygus* bugs.

When mature, the seeds were harvested and kept at room temperature. Subsequently, all of the seeds of a given umbel or of a group of umbels of a particular treatment collected on a given date were thoroughly mixed and representative samples of 50 seeds each in duplicates were examined for embryolessness by a technique previously described (5). The data obtained are presented below.

RESULTS

Early in the summer of 1949, potted dill plants were transferred from the greenhouse to outside insect-free cages when the primary umbel (first order) on each plant was showing signs of development. Subsequently, umbels of the second, third, and sometimes of the fourth order developed.

In one experiment, *Lygus* bugs were added to cages containing plants with the various orders of umbels at different stages of development ranging from flowering to firm brown seeds. The umbels were removed as they ripened and the period of exposure for the individual umbels thus varied according to the stage of seed development when *Lygus* were added. In the other experiment, a different set of plants was caged and *Lygus* were added when umbels of the first order had flowered and the ovaries were swelling. Subsequently, only these umbels were collected. The seeds all ripened at about the same time and the period of exposure to *Lygus* was approximately 25 days. The results obtained from these two experiments appear in Table I. An ample supply of *Lygus* bugs was maintained in the respective cages while the control plants were kept free of *Lygus*.

After the ripe umbels were harvested, two samples of seed from each were examined for embryolessness and the summarized data are presented in Table I. The average percentage of embryoless seeds produced when *Lygus* bugs were present was 51 and 67 per cent, respectively, while the controls averaged less than 1 per cent. This is similar to the data obtained during the summer of 1948 (3). In both years a few embryoless seeds occasionally were obtained in the controls and it is not known whether this is

due to some other factor or insect or whether *Lygus*, perhaps as nymphs, entered the control cages. However, when *Lygus* were intentionally added large percentages of the seeds were embryoless. It was shown in the earlier study (3) that nymphs as well as adults were destructive. When allowed to feed on developing dill seeds these insects were able to destroy the embryo at almost any stage of development but caused no apparent damage to either the endosperm or the fruit coats.

Lygus bugs were also caged with plants of other umbelliferous species and the data obtained on embryolessness are shown in Table II. When the experiment was well under way and seeds were developing, *Lygus* bugs

TABLE I

EFFECT OF EXPOSING DILL AT VARIOUS STAGES OF FLOWERING AND SEED DEVELOPMENT TO FEEDING BY LYGUS OBLINEATUS

Insects added	Stage of seed development	No. of plants	No. of umbels	Seed produced		
				Total No.	Embryoless, %	
					Average	Range
Lygus	Flowering to firm brown seed	10	57	28,053	51	2– 94
None		10	52	22,185	0.6	0– 2
Lygus	Past flowering, ovaries swelling	16	16	818	67	8–100
None		13	13	2,296	0.2	0– 3

were detected in several of the control cages. A number of these insects were found in the wild carrot cage feeding on several umbels containing seeds well set but not yet ripe and it is not known how they entered. Five *Lygus* bugs were inadvertently added to the cage containing the control parsley and cultivated carrot plants. While this error was discovered within 24 hours the insects could have produced considerable damage during this period. As soon as detected, the *Lygus* bugs were removed and the control plants of wild and cultivated carrot and parsley as well as the inside of the cages were sprayed with TEPP and this procedure was repeated at periodic intervals thereafter. The control cages and plants for all the other umbelliferous species were henceforth similarly sprayed.

As seen in Table II embryolessness was obtained in the carrot and parsley controls but higher percentages were produced in the *Lygus* cages. The presence of these insects in the control cages could readily account for the percentage of embryoless seeds obtained since it was previously shown (3) that just one *Lygus* bug, whether adult or nymph, could produce a tremendous amount of destruction in a very short period of time.

Although no *Lygus* bugs were observed on the control celery plants, 8 per cent embryolessness was obtained as compared with 61 per cent

TABLE II

PRODUCTION OF EMBRYOLESS SEEDS IN VARIOUS UMBELLIFEROUS SPECIES

Potted plants, outside, summer 1949		Seed production			
Kind	Treatment	Total number			Embryoless, %, Av.
		Plants	Umbels	Seeds	
Carrot, cultivated	Caged insect-free*	5	13	1,292	3*
	Caged with *Lygus*	4	3	419	50
Carrot, wild	Caged insect-free*	5	16	2,478	20*
	Caged with *Lygus*	3	8	2,750	36
	Open field	1	1	1,549	2
Celery	Caged insect-free	2	89	1,841	8
	Caged with *Lygus*	2	61	1,399	61
Coriander	Caged insect-free	2	2	52	0
	Caged with *Lygus*	4	4	99	62
Fennel	Caged insect-free	8	21	6,201	0
	Caged with *Lygus*	6	14	5,233	73
	Open field	10	11	7,346	4
Goutweed	Caged insect-free	2	5	139	0
	Caged with *Lygus*	2	1	235	52
Parsley	Caged insect-free*	2	5	61	1*
	Caged with *Lygus*	2	13	232	100
	Open field	2	12	72	5
Parsnip	Caged insect-free	9	75	5,061	0
	Caged with *Lygus*	10	50	2,559	16
	Open field	5	17	5,336	8

* During the experiment *Lygus* were found and removed from control cages.

TABLE III

SEED PRODUCTION OF DILL AND PARSNIP WHEN CAGED WITH LYGUS OBLINEATUS CONTINUOUSLY FROM THE PRE-FLOWERING STAGE TO MATURE SEED STAGE

Potted plants, outside*			Harvest			
Kind	Treatment	No.	Umbels		Seeds, No.	
			Kind	No.	Total	Av. per plant
Dill	Caged insect-free	16	First order	16	3,878	242
	Caged with *Lygus*	8		8	0	0
	Open field	8		8	1,772	222
Parsnip	Caged insect-free	9	All orders	50	1,568	173
	Caged with *Lygus*	9		52	4	0
	Open field	1		7	626	626

* Experiment started June 29, 1949.

produced in the cages when *Lygus* were maintained in the treated cages throughout the duration of the experiment. As in the case of carrot all of the celery plants were grown in the open until the time of caging. It is not known whether eggs or undetected insects were present at that time or whether the sprayings undertaken when the experiment was started and resumed sometime later were inadequate control measures.

TABLE IV

EFFECT OF CAGING VARIOUS INSECTS WITH DEVELOPING SEEDS OF DILL AND FENNEL ON THE PRODUCTION OF EMBRYOLESS SEEDS

Potted plants, caged outside, summer 1949						Harvest		
Insects added							Mature seed	
Order	Family	Species	Feeding habits	Seed development, stage	Species	Umbel No.	Total No.	Condition
Coleoptera	Chrysomelidae	Spotted cucumber beetle	Chewing	Ovaries swollen	Fennel	1	181	Embryo normal, endosperm scant
		Smartweed flea beetle		Ovaries swollen	Dill	1	201	Embryo normal, endosperm scant
				Ovaries swollen	Fennel	1	120	Embryo normal, endosperm scant
				Embryo immature, endosperm mature		1	269	Apparently normal
Hemiptera	Pentatomidae	Stinkbug (sp. ?)	Sucking, piercing	Ovaries swollen	Dill	2	0	—
				Mature		2	67	About 50% with endosperm gray and punctured
				Ovaries swollen	Fennel	1	0	—
	Miridae	Tarnished plant bug (*Lygus*)		Ovaries swelling	Dill	1	106	84% Embryoless
				Various stages		1	584	63% Embryoless
				Ovaries swollen	Fennel	2	1016	79% Embryoless
				Embryo immature, endosperm mature		1	298	88% Embryoless

It is not likely that TEPP would destroy the eggs since it hydrolyzes so rapidly and has little or no residual effect. During the experiment the celery plants were frequently sprayed with nicotine because of heavy aphid infestations and it is possible that some of the very small insects observed were *Lygus* nymphs.

In coriander, fennel, goutweed, and parsnip seeds (Table II) the average percentage of embryolessness ranged from 16 to 100 per cent in the *Lygus* cages while all the control seeds contained embryos. Since *Lygus*

bugs are present in Yonkers—although not in large numbers—it is to be expected as seen in Tables II and III that some embryoless seeds would be found on uncaged plants in the open field.

Since some indication was obtained in the 1948 studies that *Lygus* bugs may reduce seed yield when feeding occurs at or near the flowering period, a more comprehensive experiment was undertaken. *Lygus* bugs were caged with plants of both dill and parsnip just prior to the flowering period and an ample supply of these insects was maintained in the cages until the seeds in the control cages were almost ripe. Although flowering occurred and houseflies were present as pollinators the resulting seed crop was a total failure as is shown in Table III. Thus *Lygus* bugs can destroy either the flower and/or seed in the very early stages of development but when feeding occurs at a later stage they destroy the immature embryo and do not disturb the more mature endosperm (Table IV).

Various chewing and other sucking insects found out-of-doors on different umbelliferous plants were collected and caged with individual umbels of dill and fennel when the seeds were at the various stages of development as listed in Table IV column six. It is readily seen that *Lygus* bugs were the only insects feeding solely on the embryo while the other insects tested either destroyed the seed entirely or attacked only the endosperm. In the earlier report (3, p. 302) other insects found on umbelliferous plants such as ants, fireflies, Japanese beetles, lace wings, ladybird beetles, and syrphid flies were also caged with dill plants but none of these produced embryoless seeds such as occur when *Lygus* bugs are present.

DISCUSSION

Sometimes as high as 50 per cent or more of a freshly-harvested crop of apparently well-filled umbelliferous seeds cannot germinate because the embryo is lacking although the endosperm appears normal. Embryolessness in this group has been found to occur at random from year to year with no correlation as to source or variety, yield, soil types, climatic conditions, genetical influence, etc. (2). However, in a previous study (3) as well as in the present one, embryolessness almost invariably occurred and usually in very high percentages on plants or specific umbels which were caged with *Lygus* bugs.

In the Umbelliferae double fertilization occurs (8). The endosperm soon becomes mature and firm while the embryo slowly undergoes changes and reaches maturity at about the time the external appearance of the seed changes from green to brown (3, p. 305). The mature embryo is quite small and lies imbedded in the endosperm, while in embryoless seed there is a cavity in the endosperm where the embryo would normally be located. Since the embryo in its development remains immature over such a relatively long period of time, the *Lygus* bugs have greater opportunity to feed upon and destroy it. When these insects, nymphs or adults, were

caged and allowed to feed on the developing seed (3) they destroyed the embryo at almost any stage of development but caused no apparent damage to either the endosperm or the fruit coats. However, when *Lygus* bugs fed on the flowers or ovaries, the entire fruit was destroyed and thereby the seed yield was reduced as shown above.[1]

Lygus bugs have been known to reduce seed yields in various other species of plants for they are rather omnivorous feeders and produce bud blasting, blossom and young fruit drop, retardation of vegetative growth (11), general dehabilitation of the plant, and partial as well as complete destruction of the seeds. Much of the literature pertaining to seeds was reviewed earlier (3). Various investigators have shown that seed yields are greatly increased when *Lygus* bugs are controlled. The results with sugar beet illustrate, for instance, that not only seed yield (7, 12) but also seed viability (9) is increased by proper control of these insects. Several large growers of umbelliferous seeds in the western states report by correspondence that embryolessness was considerably reduced in 1949 in the fields which were dusted primarily to control *Lygus* bugs.

In all the studies to date with umbelliferous seeds *Lygus* bugs consistently produce high percentages of embryolessness when caged with plants or specific umbels. When various other kinds of insects found on umbelliferous plants were caged with umbels of dill and fennel, in some cases nothing happened (3, p. 302) while in other cases (as seen above) the seeds were either entirely destroyed or the endosperm was attacked while the embryo remained intact. While thus far only *Lygus* bugs have been found to produce embryolessness in this family, it is possible that other insects or perhaps some cultural condition may produce worthless seed.

In the data presented above, although *Lygus* bugs were found in the control cages of carrot and parsley and could very easily account for the percentage of embryolessness obtained, it is not known whether this was the sole contributing factor. While no insects were found on the control celery plants, it is difficult to detect the nymphs (through the glass or mesh screening on the cages), due in part to their small size and in part to their coloration which is very similar to that of the plant. In addition, celery produces umbels, leaves, and branches in great profusion which all serve as excellent hiding places for *Lygus* which even as adults are not large (about 5 mm. in length). It is known that *Lygus* bugs oviposit in celery in early June as well as later and that as a result large broods of nymphs subsequently hatch (6). Many nymphs have been reported on wild carrot in late summer (1) and nymphs of two *Lygus* species have been

[1] *Note added in galley proof:* Handford, R. H. [*Lygus campestris* (L.): a new pest of carrot seed crops. Canadian Ent. **81**: 123–126. 1949] has reported that *Lygus campestris* seriously reduced carrot seed yield and when adequately controlled increases in seed yield were obtained.

observed on cultivated carrot (10). Thus the source of *Lygus* noted above on the wild carrot controls may have been due to hatchings of eggs oviposited prior to the time of caging the plants.

SUMMARY

Various insects such as *Lygus* bugs, stinkbugs, flea beetles, and cucumber beetles found on umbelliferous plants were collected and caged with developing seeds of dill and fennel. Only *Lygus* bugs produced embryolessness, that is, seeds with a cavity instead of an embryo while the endosperm and fruit coats remained apparently normal. *Lygus* bugs also produced embryolessness in other umbelliferous species such as carrot, celery, coriander, goutweed, parsley, and parsnip. It has not yet been determined whether other factors or insects can also produce embryoless seeds in this family; however, the studies to date suggest that *Lygus* bugs are primarily responsible for the natural occurrence of the high frequency of this condition.

In addition to producing embryolessness, *Lygus* bugs can greatly reduce the seed yield in the Umbelliferae since the dill and parsnip crops were a total failure when insects were present during and immediately following the flowering period. Feeding at a later stage of seed development when the endosperm is mature but the embryo is still immature results in embryolessness.

LITERATURE CITED

1. CROSBY, CYRUS RICHARD, and MORTIMER DEMAREST LEONARD. Manual of vegetable-garden insects. 391 pp. Macmillan Co., New York. 1918.
2. FLEMION, FLORENCE, and ESTHER THAYER HENRICKSON. Further studies on the occurrence of embryoless seeds and immature embryos in the Umbelliferae. Contrib. Boyce Thompson Inst. **15**: 291–297. 1949.
3. FLEMION, FLORENCE, HARRIET POOLE, and JUNE OLSON. Relation of *Lygus* bugs to embryoless seeds in dill. Contrib. Boyce Thompson Inst. **15**: 299–310. 1949.
4. FLEMION, FLORENCE, and GLORIA UHLMANN. Further studies of embryoless seeds in the Umbelliferae. Contrib. Boyce Thompson Inst. **14**: 283–293. 1946.
5. FLEMION, FLORENCE, and ELIZABETH WATERBURY. Embryoless dill seeds. Contrib. Boyce Thompson Inst. **12**: 157–161. 1941.
6. HILL, L. L. Further studies of tarnished plant bug injury to celery. Jour. Econ. Ent. **26**: 148–150. 1933.
7. HILLS, ORIN A., and EDGAR A. TAYLOR. *Lygus* damage to beet seed in varying stages of development. Proc. Amer. Soc. Sugar Beet Technol. **5**: 502–503. 1948.
8. JURICA, HILARY STANISLAUS. A morphological study of the Umbelliferae. Bot. Gaz. **74**: 292–307. 1922.
9. MORROW, W. B. Effect of commercial *Lygus* control on seed viability in Arizona and New Mexico. Proc. Amer. Soc. Sugar Beet Technol. **5**: 494–495. 1948.
10. SHULL, WESLEY EARL. An investigation of the *Lygus* species which are pests of beans (Hemiptera, Miridae). Idaho Agric. Exp. Sta. Res. Bull. 11. 42 pp. 1933.
11. STITT, LOYD L. Reduction of the vegetative growth of alfalfa by insects. Jour. Econ. Ent. **41**: 739–741. 1948.
12. WOOD, R. C. Effect of *Lygus* control on seed yields in Arizona and New Mexico. Proc. Amer. Soc. Sugar Beet Technol. **5**: 495–496. 1948.

BIOASSAY OF INSECTICIDE SPRAY RESIDUES IN PROCESSED FOOD[1]

ALBERT HARTZELL AND ELEANOR E. STORRS

INTRODUCTION

Public interest in the importance of spray residues is at the highest level since the arsenic scare in the 1920s. The hazard to health has increased greatly with the advent of such insecticides as benzene hexachloride, parathion, and chlordan which are many times more toxic to animals than DDT. The hearings now in progress before the Food and Drug Administration will lead to the establishment of minimum tolerances for most of the insecticides and fungicides used on crops.

This will throw a great burden of responsibility on the processors of food. They will have to know what materials were used on the crop, what concentrations persist at harvest, and whether these materials will persist throughout washing and processing operations. This is no small assignment in view of the dozens of new compounds on the market and many others in the developmental stage. Obviously there is a great need for simple, practical methods of analyzing for these substances, and for information on the fate of the different insecticides at each stage of processing. The present paper deals with a single phase of this larger problem, namely the detection of insecticides in processed foods.

Several chemical methods have been developed for the determinations of small amounts of certain residues from these new synthetic insecticides. These methods are for the most part colorimetric procedures or are based upon the analyses of organically bound chlorine. Such chemical methods conceivably may suffer from lack of specificity in that they may in some cases measure breakdown products derived from the insecticides instead of the actual insecticides themselves.

Mosquito larvae are usually extremely sensitive toward insecticides and thus are well adapted for use in bioassay as has been pointed out in a previous publication (4). The mosquito larvae method measures toxicity. Unless the previous history of the sample is known it is not possible to arrive at any conclusion as to the amount of any particular insecticide residue that may be present.

Nolan and Wilcoxon (5) have perfected a method of bioassay for parathion in plant material which involves extraction of the parathion with benzene, evaporation of the benzene from aliquot portions, resuspension of the residues in water, and testing with *Aedes aegypti* larvae. This method

[1] The data in this paper were obtained cooperatively with the Beech-Nut Packing Co., Canajoharie, N.Y.

Copyright, 1950, Boyce Thompson Institute for Plant Research, Inc.

is sensitive to about 0.01 p.p.m. parathion in plant material, and gives results that compare favorably with those given by the chemical method of Averell and Norris (1). It has been used mainly for the determination of parathion spray residues on fresh vegetables.

For the work reported in the present paper a general procedure similar to that of Nolan and Wilcoxon (5) was applied for the bioassay of various other insecticides as well as parathion in processed foods. Of the insecticides tested, which are listed in Table I, parathion is by far the most active against *Aedes aegypti* larvae. There are certain factors that limit the sensitivity of the bioassay. It is necessary to extract the insecticide from the plant material with an immiscible organic solvent, such as benzene, in order to avoid the gross interference due to the various plant substituents. However, the benzene extracts contain besides the insecticide other extracted materials such as fats, oils, waxes, essential oils, pigments, etc.

It was found that these other extracted materials interfere with the bioassay by reducing the toxic action of the insecticides. Also at higher concentrations these other extracted materials themselves appear to kill the mosquito larvae. Thus it is obvious that for the purpose of bioassay it would be ideal to be able first to separate the insecticide more carefully from the other extracted materials. However, this provides problems for future research. The data presented in this paper were obtained by the methods thus far developed. At the first stage of the work the toxicities of the pure insecticidal compounds in water solution were determined.

MATERIALS AND METHODS

Yellow fever mosquito larvae[2] (*Aedes aegypti* L.) were reared by methods similar to those used in the culture of *Anopheles* mosquito larvae (2, 3, 4). Eggs[3] of *Aedes aegypti* were placed in shallow porcelain pans containing tap water maintained at a depth of about one inch. The eggs hatched in about 24 hours at room temperature. One hundred mg. of ground Milk-Bone dog biscuit per liter of water were added daily. Three-day-old larvae were used in the tests. At the end of the tests the larvae were killed by covering the surface of the water with kerosene for 24 hours before disposal.

The processed foods were provided by Beech-Nut Packing Co. These were in sealed jars of the regular commercial type containing strained processed foods. The two products reported on in this paper are green beans and mixtures of apricots and apples. The proportion of apricots to

[2] In regions where adults of this species may serve as a disease vector another species of *Aedes* should be substituted. *Aedes* eggs are more convenient than eggs of *Culex* because they can be stored dry for several weeks without losing their viability.

[3] Shipped by air mail from Orlando, Florida, courtesy of C. H. Bradley.

apples was 2 to 1. Some of the jars contained produce to which known amounts of insecticides had been added in a pilot plant prior to processing. Other jars (checks) for use in control work contained foods that had not been treated with any insecticide.

TABLE I

INSECTICIDES ADDED IN KNOWN AMOUNTS TO PROCESSED FOOD

Insecticide	Active ingredient
Parathion	*O,O*-Diethyl *O*-*p*-nitrophenyl thiophosphate
Heptachlor	1 (or 3a),4,5,6,7,8,8-Heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene
Aldrin	1,2,3,4,10,10-Hexachloro-1:4,5:8-dimethano-1,4,4a,5,8,8a-hexahydronaphthalene
Dieldrin	1,2,3,4,10,10-Hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1:4,5:8-dimethanonaphthalene
Chlordan	1,2,4,5,6,7,8,8-Octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene
Lindane	Gamma isomer of benzene hexachloride (99% pure)
BNP*	2-Nitro-1,1-bis(*p*-chlorophenyl)propane
BNB*	2-Nitro-1,1-bis(*p*-chlorophenyl)butane
DDT	2,2-Bis(*p*-chlorophenyl)-1,1,1-trichloroethane
Methoxychlor	2,2-Bis(*p*-methoxyphenyl)-1,1,1-trichloroethane (100% pure)
Rhothane	2,2-Bis(*p*-chlorophenyl)-1,1-dichloroethane
Toxaphene	Chlorinated camphene, approximate formula $C_{10}H_{10}Cl_8$
Neotran	Bis(*p*-chlorophenoxy)methane
Miticide #2**	80% Butoxypolypropylene glycol + 20% Tergetol penetrant
DMC†	Bis(*p*-chlorophenyl)methyl carbinol
Arathane	Dinitrocaprylphenyl crotonate

* Commercial Solvents Co.
** Carbide and Carbon Chemicals Division, Union Carbide and Carbon Corp.
† Sherwin Williams Co.

The insecticidal compounds (Table I) used for making the standard solutions were of pure grade. Standard aqueous solutions or emulsions were prepared by adding to water, with shaking, 10 ml. or less of a standard 0.1 per cent solution in acetone of the insecticide so as to make a volume of one liter. The toxicities to the mosquito larvae of the pure insecticidal compounds in tap water were first determined. The acetone solution was added to water so as to make a 1.0 part per million emulsion. This and several serial dilutions made from it were tested on mosquito larvae.

In working with the processed foods the procedure was as follows. A weighed sample of the processed food (50 to 100 g.) was introduced by means of a wide-stemmed funnel into a bottle fitted with a ground glass stopper. One ml. of tap water and 2 ml. of benzene per gram of sample were added, the bottles were securely stoppered, and then shaken on a shaking machine for four hours. For the tests in which the insecticide was added to the control food just prior to analysis, a standard aqueous suspension of an insecticidal compound was added in the amount of 1 ml. per g. of sample and the mixture shaken for one hour. Then 2 ml. of benzene per g. of the original sample were added and the mixture shaken four hours more. In each case the resulting emulsion was broken by adding sodium sulfate, and then after standing, as much as possible of the benzene layer was decanted. A graduated series of amounts of this benzene extract (2 ml. now contained the extracted material from 1 g. of sample) was measured into beakers and the benzene was evaporated in a chemical hood without the application of heat with the aid of a current of compressed air. The residue in each beaker was redissolved by adding 0.5 ml. acetone, and then 24.5 ml. of tap water were added with stirring. Into each of duplicate test tubes 7.5 ml. of a resulting emulsion were transferred from a beaker, then 10 mosquito larvae together with sufficient additional tap water were added to make a total volume of 15 ml. The tubes were held in an oven at $30° \pm 1°$ C. for 24 hours. At the end of this period the percentage mortality was determined for each dilution. The breaking point range of this toxicity curve, that is, the range at which the kill fell from 100 per cent to less than 80 per cent, was estimated. These estimates were made on the assumption that none of the originally added insecticide had undergone destruction. When the apparent breaking point range for the food to which the insecticide had been added before processing was a very considerably higher figure than the breaking point range for the control, this indicated that some destruction of the insecticide had occurred in the processed food.

Comparative tests were performed on three types of extracts, namely (a) benzene extracts of check foods to which graduated amounts of a standard acetone solution of an insecticide were added after evaporating the benzene, (b) benzene extracts of a recently shaken mixture of check foods and aqueous emulsion of known amount of an insecticide, and (c) benzene extracts of foods to which a known amount of an insecticide had been added before processing.

Since mosquito larvae are sensitive to very minute amounts of insecticide, cleaning the glassware presents a problem. The glassware was first rinsed with acetone, then placed in hot trisodium phosphate solution to soak overnight. The glassware was then successively rinsed with hot running tap water, chromate-sulfuric acid cleaning solution, and finally thoroughly rinsed in hot running tap water.

RESULTS AND DISCUSSION

Results of testing water solutions of pure insecticides. The results of these tests appear in Table II. The data show that parathion is by far the most active insecticide against the mosquito larvae. It caused 100 per cent mortality even at the low concentration of 0.0006 p.p.m. Most of the other insecticides gave complete kills at 0.10 to 0.01 p.p.m. The last four materials listed in Table II are of such low toxicity to mosquito larvae that they probably cannot be assayed by the use of this test organism.

TABLE II

MORTALITY OF MOSQUITO (AEDES AEGYPTI) LARVAE IN AQUEOUS SUSPENSION OF SIXTEEN INSECTICIDES AT DIFFERENT CONCENTRATIONS

Insecticide	Mortality of larvae in per cent killed at various concns. (in p.p.m.)												
	1.0	.5	.25	.125	.0625	.031	.016	.0078	.005	.0025	.00125	.0006	.0003
Parathion		100	100	100	100	100	100	100	100	100	95	100	0
Heptachlor	100	100	100	100	100	100	100	45	35	0	0		
Aldrin	100	100	100	100	100	100	100	100	65	65	0		
Dieldrin	100	100	100	100	100	100	100	65	10	15	5		
Chlordan	100	100	100	100	100	100	100	15					
Lindane	100	100	100	100	100	100	75	65	0				
BNP*		100	100	100	100	95	80	90	45	20			
BNB*		100	100	100	45	0	0	0	0	0	0		
DDT		100	100	100	100	65	15	0	0	0	0		
Methoxychlor	100	100	100	100	100	35	0	0					
Rhothane	100	100	95	100	100	80	65	30					
Toxaphene	100	100	100	100	100	85	0	0					
Neotran		100	25	0	0	0	0	0	0	0	0		
Miticide #2**		0	0	30	0	0							
DMC†		0	0	0	0	0							
Arathane		45	45	0	0	0							

*,**,† (See footnotes Table I).

Results obtained with the benzene extraction method. The results are shown in Table III. Appropriate reference standards for this assay must contain the other extracted materials from the food as well as the extracted insecticide. In the fourth column are given the results for the standards used for comparisons with the results in the fifth and sixth columns. Standard solutions which did not differ greatly from the test solutions in respect to the ratio of the insecticide to the extracted food material were compared. The breaking point range of the standards was selected from such comparisons, as indicated in Table III. That these extracted materials have an effect in reducing the toxicity of at least some insecticides is quite evident from a comparison of the data from the standards in Table III with the data in Table II. This is particularly marked in the case of chlordan, Rhothane, and toxaphene. In some of the other standard tests (not shown in the tables) in which the amounts of extracted material from the check foods were considerably greater, the breaking point range

TABLE III

TOXICITY TO MOSQUITO LARVAE OF INSECTICIDES IN EXTRACTS OF PROCESSED FOODS PREPARED THROUGH BENZENE EXTRACTION

Insecticide	Amount added to food, p.p.m.	Food*	Breaking point range, p.p.m.**		
			Standard***	Added after processing	Added before processing
Heptachlor	5.0	B	0.016–0.004	0.06 –0.03	>1.0†
	5.0	A	0.06 –0.016	0.06 –0.03	1.0 –0.5
Aldrin	5.0	B	0.06 –0.016	0.06 –0.03	>0.06 (50% kill)
	5.0	A	0.06 –0.016	>0.06 (50% kill)	>0.06 (70% kill)
Dieldrin	5.0	B	0.016–0.004	0.03 –0.016	0.016–0.008
	5.0	A	0.016–0.004	0.03 –0.016	0.03 –0.016
Dieldrin	0.5	B	0.013–0.003	0.03 –0.006	0.03 –0.013
	0.5	A	0.013–0.003	0.03 –0.006	0.03 –0.013
Chlordan	5.0	B	0.5 –0.125	0.5 –0.25	>1.0
	5.0	A	0.5 –0.125	1.0 –0.25	1.0 –0.5
Lindane	5.0	B	0.016–0.004	0.03 –0.016	0.25 –0.06
	5.0	A	0.016–0.004	0.016–0.008	0.03 –0.01
Lindane	1.0	B	0.013–0.003	0.05 –0.025	0.1 –0.05
	1.0	A	0.013–0.003	0.05 –0.025	0.05 –0.025
BNP††	5.0	B	0.03 –0.008	0.06 –0.03	0.125–0.06
	5.0	A	0.125–0.03	0.06 –0.03	0.06 –0.03
BNB††	5.0	B	0.125–0.03	0.25 –0.125	0.5 –0.25
	5.0	A	0.125–0.03	0.06 –0.03	0.25 –0.125
Methoxychlor	5.0	B	0.25 –0.06	0.5 –0.125	>1.0
	5.0	A	0.25 –0.06	0.25 –0.125	0.5 –0.25
Rhothane	5.0	B	0.5 –0.125	0.125–0.06	>0.5 (80% kill)
	5.0	A	0.5 –0.125	0.25 –0.125	0.25 –0.125
Toxaphene	5.0	B	1.0 –0.25	>1.0	>1.0
	5.0	A	1.0 –0.25	>1.0	>1.0

* B = Beans; A = apricots and apples.

** The breaking point ranges from 100% to less than 80% kill. The indicated breaking point range is based on the assumption that there had been no destruction of the added insecticide. Actually some destruction had occurred in some instances, as can be seen from the data.

*** The standard was diluted serially by fourths while the others were diluted by halves, thus the wider breaking point ranges in the standard.

† The highest concentrations tested did not give 100% kill, so the highest concentration is shown, indicating that the upper limit of the breaking point was not reached.

†† Commercial Solvents Co.

usually came at the lower concentration of the insecticide, presumably due to the toxic action of the other extracted materials themselves. This fact necessarily implies some limitations on the present bioassay method. For this reason the breaking point ranges are recorded in this paper rather than the more exact LD_{50} values.

The foods that had the insecticides added after processing just prior to assay gave breaking point ranges which were not greatly different from the untreated controls.

The foods that had the insecticide added before processing showed definite partial destruction of the insecticide in the case of heptachlor in both beans and apricots and apples, and methoxychlor and chlordan in beans. No definite destruction was noted in the case of dieldrin in either beans or apricots and apples and BNP, Rhothane, aldrin, and chlordan in apricots and apples.

SUMMARY

Known amounts of spray residues of ten insecticides were bioassayed in processed foods (strained beans, and mixtures of apricots and apples) by a modified Nolan and Wilcoxon method for parathion, involving the extraction of the insecticide from the food product with benzene, evaporating the benzene with compressed air, and testing the extract in water on *Aedes aegypti* mosquito larvae for toxicity.

The insecticides assayed by this method were heptachlor, aldrin, dieldrin, chlordan, lindane, BNP, BNB, methoxychlor, Rhothane, and toxaphene.

The various insecticides were readily detected when added to processed foods at concentrations of 0.5 to 5.0 p.p.m. Benzene extracts of such samples were diluted to a dosage equivalent to 0.01 to 1.0 p.p.m. for critical assaying. The critical dilution of extracts from aqueous suspensions was to a concentration of 0.125 to 0.01 p.p.m.

In general, the insecticide toxicity was inhibited when combined with plant material. Strained beans inhibited toxicity to some insecticides more than a mixture of strained apricots and apples.

Heptachlor in both beans and apricots and apples, and methoxychlor and chlordan in beans showed partial destruction in processing.

LITERATURE CITED

1. AVERELL, P. R., and M. V. NORRIS. Estimation of small amounts of *O,O*-diethyl *O,p*-nitrophenyl thiophosphate. Analytical Chemistry **20**: 753–756. 1948.
2. DEONIER, C. C., E. HINCHEY, and H. H. INCHO. Evaluation of materials as mosquito larvacides. *In* Results of screening tests with materials evaluated as insecticides, miticides and repellents at the Orlando, Fla., Laboratory. April 1942 to April 1947. U.S. Dept. Agric. Bur. Entomol. & Plant Quar. E-733, 7–9. 1947.
3. GRANETT, PHILIP, and HARRY L. HAYNES. Improved methods of rearing *Aedes aegypti* mosquitoes for use in repellent studies. Proc. New Jersey Mosquito Exterm. Assoc. **31**: 161–168. 1944.
4. HARTZELL, ALBERT. Toxicity of spray residue of fresh and processed fruits and vegetables. *In* Agric. Control Chemicals. Collected papers from the Symposia on Economic Poisons presented before the Division of Agric. and Food Chem., Amer. Chem. Soc. Advances in Chem. Series **1**: 99–101. 1950.
5. NOLAN, KENNETH, and FRANK WILCOXON. Method of bioassay for traces of parathion in plant material. Agric. Chemicals **5** (1): 53, 74. 1950.

RELATION OF DIFFERENT GASES TO THE SOAKING INJURY OF SEEDS

Lela V. Barton

Seeds of *Phaseolus* are injured by soaking in water (3, 4, 8, 9, 11, 14). It might be assumed that such injury was due to anaerobic conditions, since oxygen is required for respiration. It has been shown, however, that exposure of oat seeds to an excess of oxygen during soaking resulted in abnormal germination (1). In order to gain more information on this subject, tests were made on the effects of passing oxygen, carbon dioxide, hydrogen and nitrogen through the soaking medium on the germination of seeds of beans, oats, and six other plants. The results confirmed the finding of Albaum that oxygen bubbling through the soaking water increased the injurious effects. They showed further that nitrogen or hydrogen so supplied reduced the harmful effects somewhat, while carbon dioxide reduced them still more or prevented them entirely.

MATERIAL AND METHODS

Seeds used for the tests to be reported here were: *Phaseolus vulgaris* L. varieties Dwarf Horticultural, Horticultural, French Horticultural, Tendergreen, and Golden Cluster Wax; *Phaseolus lunatus* L. variety Bush Lima; *Vicia faba* L. variety English Windsor; *Pisum sativum* L. variety Perfection; *Helianthus annuus* L.; *Xanthium canadense* Mill.; *Avena sativa* L. varieties Clydesdale and Siegis-Hafers; *Hordeum vulgare* L.; *Triticum aestivum* L. variety Marquis; and *Zea mays* L., Pioneer Hi-Bred X4758A.

The usual procedure was to soak counted lots of seeds in a measured amount of tap water at laboratory temperature (20° to 26° C.) in flasks through which gases were bubbled slowly for a period of 24 hours. At the termination of the soaking period the water was drained off and the seeds were divided into lots for germination tests on moist filter paper or paper towels in controlled temperature cabinets or in soil under greenhouse conditions. *Phaseolus*, *Vicia*, *Helianthus*, and *Zea* were germinated in cabinets held alternately at 20° C. for 16 hours and 30° C. 8 hours. *Avena*, *Hordeum*, and *Triticum* were germinated at a constant temperature of 20° C. and *Xanthium* at 30° or 35° C. Duplicate lots of 25 seeds each were used for planting except for *Avena*, *Hordeum*, and *Triticum* in which cases duplicates of 50 were used.

The effect of time of soaking, the moisture content of the germination medium, and gas mixtures on subsequent germination were determined. Also moisture absorption rates were measured during the soaking exposure of bean seeds to different gases. Experiments were also carried out to ascertain the relative effects of the gases on intact seeds and ex-

Copyright, 1950, by Boyce Thompson Institute for Plant Research, Inc.

cised embryos. Records were taken of germination performance, the vigor and appearance of the resultant seedlings being noted.

Two types of controls were used, dry seeds and seeds which had been soaked in non-aerated water. Also, air bubbling through the soaking water might be considered a third control. Air was usually forced through the cultures by vacuum, or more rarely by compression. In order to assure an atmosphere of the gas at the surface of the water during the soaking process, a closed system where the gas was bubbled through water after leaving the soaking solution was used in most of the tests.

EXPERIMENTAL RESULTS AND DISCUSSION

RESPONSE OF DIFFERENT SPECIES

In Table I are shown germination percentages secured from seeds soaked in tap water in the laboratory for 24 hours. For all of the beans used, the harmful effect of soaking in non-aerated water is evident; ranging from a small reduction in subsequent germination for the Tendergreen variety (from 100 to 82 per cent) to complete inhibition of germination for Golden Cluster Wax. When air was supplied during the soaking process, slightly less injury resulted, but oxygen caused a great reduction in germination

TABLE I

EFFECT OF SOAKING VARIOUS SEEDS IN TAP WATER THROUGH WHICH DIFFERENT GASES WERE BUBBLED FOR A PERIOD OF 24 HOURS BEFORE PLANTING

Species	Variety	Percentage germination in incubators or greenhouse after soaking									
		Incubators					Greenhouse				
		Dry	Soaked				Dry	Soaked			
			No gas	Air	O_2	CO_2		No gas	Air	O_2	CO_2
Phaseolus vulgaris	Dwarf Horticultural	96	24	60	0	92	92	90	84	0	96
	Tendergreen	100	82	98	16	100	86	78	86	0	90
	Golden Cluster Wax	94	0	12	0	94	96	22	48	0	98
Phaseolus lunatus	Bush Lima	90	15	40	15	80	90	75	50	0	70
Pisum sativum	Perfection	—	—	—	—	—	83	93	98	16	94
Vicia faba	English Windsor bean	20	20	20	0	40	65	75	35	0	55
Helianthus annuus	Sunflower	98	90	88	74	78	98	100	100	98	98
Avena sativa	Clydesdale oats	98	98	90	13	75	98	98	100	58	88
	Siegis-Hafers oats	76	39	40	14	5	89	84	81	51	63
Triticum aestivum	Marquis wheat	100	98	99	99	100	98	96	99	89	99
Zea mays	Pioneer Hi-Bred corn	100	98	96	80*	88	100	96	96	72	90

* 48% Abnormal; 32% normal.

of all varieties. Carbon dioxide, on the other hand, prevented the harmful effect of the air and oxygen and even of the soaking process itself as manifested in the non-aerated solutions. That this is not due solely to the exclusion of oxygen has been demonstrated in numerous tests where nitrogen or hydrogen was used instead of carbon dioxide. Some of the data from these tests will be shown later, but the nature of the effects may be

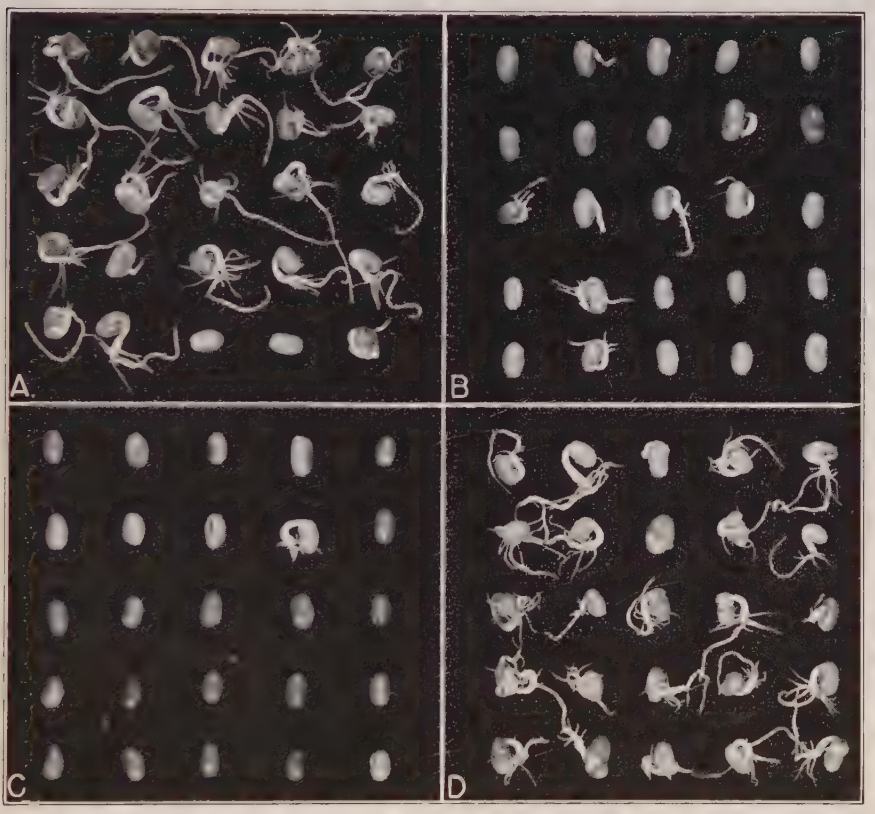


FIGURE 1. Germination of Dwarf Horticultural beans in rolled moist paper towels following soaking in the laboratory for 24 hours. A. Dry control. B, C, and D. Soaked in tap water through which nitrogen, oxygen, and carbon dioxide, respectively, were bubbled.

seen in Figure 1. The seeds are in much better condition after receiving a supply of nitrogen during the soaking than after oxygen treatment, but nitrogen does not permit the capacity or vigor of germination evident in the dry controls or after carbon dioxide treatment.

As far as we know, beneficial effects on seeds of carbon dioxide supplied during the soaking process have not been reported before. It is at variance with data obtained by Kisser and Possnig (11). In studies with seeds of *Triticum*, *Pisum*, and *Vicia*, they found that passing air through soaking

water stimulated germination; that the results were poorer with hydrogen and nitrogen; and that there was some injury with carbon dioxide. They attributed the injury to peas with more than four hours' soaking in aerated water to an accumulation of carbon dioxide.

Other work on the physiological effects of carbon dioxide have utilized a moist substratum for seeds in an atmosphere with an augmented carbon dioxide supply. Under these conditions Kidd (8) obtained results which led him to believe that the resting stage of the moist seed is a type of narcosis induced by carbon dioxide pressure in the tissues of the embryo and that the inhibiting value of a given carbon dioxide pressure dimishes with a rise in temperature or oxygen pressure. Thornton (12, 13), on the contrary, was able to break the dormancy of lettuce and cocklebur seeds under certain conditions by increasing the pressure of carbon dioxide. It should be emphasized that both Kidd and Thornton used moist substrata and did not soak the seeds. The gas effects as described in the present tests depend upon the immersion of seeds in water for their manifestations. Bean and oat seeds, both dry and presoaked in non-aerated water for eight hours, were placed on moist filter paper in aeration flasks and oxygen or carbon dioxide was passed over them for 24 and 48 hours with no resultant evident oxygen injury to germination. There was, however, some indication of decreased speed and percentage of germination of bean seeds which had been exposed to carbon dioxide for 48 hours under these conditions.

It will be noted from Table I that none of the oxygen-treated bean seeds were capable of producing seedlings in soil in the greenhouse. On the contrary, certain varieties, such as Dwarf Horticultural and Bush Lima for example, from the non-aerated control or the air treatment germinated better in soil in the greenhouse than when rolled in moist paper towels and placed at an alternation of 20° to 30° C. Examples of this behavior were found in other tests and may be related to the amount of moisture in the germination medium. Some data on this phase will be presented below. There is some indication that seeds of *Pisum sativum* were benefited by soaking except when oxygen was supplied. This may have been due to the resistance of the seed coats to the absorption of water.

Injury as a result of soaking in water saturated with oxygen was also demonstrated for oat seeds (Table I). Clydesdale oats were more resistant to soaking injury per se than Siegis-Hafers. This may have been due to the poorer quality of the latter. Also, it will be noted that carbon dioxide not only failed to prevent the harmful effects of soaking for this grain, but actually reduced germination. The same effect is to be seen for wheat and corn. Under the conditions of this experiment, only slight injury to wheat resulted from treatment with oxygen, while corn occupied an intermediate position between oats and wheat.

EFFECT OF MOISTURE CONTENT OF GERMINATION MEDIUM

It was noted early in the experiments that too much moisture on the filter paper or paper towels used for germination of soaked seeds tended to lower the percentages obtained. To ascertain the best moisture condition, tests were made using dry, medium wet, and wet substrates. Thirty-six Scot Tissue towels were moistened with 300, 550, and 900 ml. of water to form dry, medium, and wet substrates respectively, to receive the bean seeds. For oats, five sheets of 15-centimeter Eaton-Dikeman filter paper No. 613 were moistened with 10, 20, and 30 ml. of water.

The results of one of these tests for Dwarf Horticultural beans and

TABLE II

EFFECT OF MOISTURE CONTENT OF FILTER PAPER OR PAPER TOWELS ON THE GERMINATION (PERCENTAGES) OF DRY AND SOAKED SEEDS OF DWARF HORTICULTURAL BEANS AND CLYDESDALE OATS

Seeds	Previous treatment		Moisture content of substrate		
			Low	Medium	High
Beans	Soaked	No gas	95	100	85
		Air	100	85	93
		O_2	38	17	5
		N_2	100	95	95
		CO_2	98	93	93
	Dry		48	48	83
Oats	Soaked	No gas	85	78	12
		Air	67	80	8
		O_2	44	18	1
		N_2	95	91	6
		CO_2	77	71	0
	Dry		96	96	96

Clydesdale oats are shown in Table II. The adverse effect of the excess moisture was strikingly demonstrated for all soaked oats and for the oxygen-treated beans. Dry seeds were not affected by the water supplied in this experiment except that germination of beans was somewhat delayed on the dry and medium wet substrates so that germination was not complete at the termination of the test.

For the remainder of the experiments reported in this paper, a medium amount of moisture was used for germination tests.

EFFECT OF DURATION OF TREATMENT

Results of the oxygen and carbon dioxide treatments of one of the experiments to determine the relation of the duration of the soaking period to injury are shown in Figure 2. Injury to oxygenated beans is apparent after six hours of soaking, the germination being reduced to 64 as com-

pared with 94 per cent after the three-hour treatment. Further soaking for 12 hours permitted only 16 per cent germination, while 24 hours killed almost all of the seeds. Seeds treated with carbon dioxide, on the other hand, showed remarkable vigor after 12 hours but did not grow so rapidly after 24 hours. In non-aerated or aerated water (not shown in photograph)

FIGURE 2. Germination of Dwarf Horticultural beans soaked in water through which oxygen (1 and 3) or carbon dioxide (2 and 4) was bubbled for 3 (A left), 6 (A right), 12 (B left), or 24 (B right) hours.

there was a gradual decrease in germination capacity with increased duration of soaking to 34 and 46 per cent respectively. Germination of the dry control was 98 per cent.

Other effects of soaking time are given in Table III, second part, for French Horticultural beans and Clydesdale oats. Dry seeds and seeds which had been presoaked for eight hours in non-aerated water were placed in water through which oxygen or carbon dioxide was bubbled for periods up to 16 hours. It will be seen that the injury is increased by the pre-

soaking though the latter was not deleterious in itself, when not followed by additional soaking. When the total time was extended to 24 hours, however (eight hours presoaking + 16 hours of gas exposure), carbon dioxide failed to prevent completely the deterioration process, which presumably was initiated during the presoaking period. This indicates that the gas effects cannot be entirely separated from the effect of the water.

TABLE III

EFFECT OF PRESOAKING AND DURATION OF GAS EXPOSURE ON SUBSEQUENT GERMINATION OF FRENCH HORTICULTURAL BEANS AND CLYDESDALE OATS

Total period of combined treatment 8 hours or less

Hours gas exposure	Hours presoaking of beans						Hours presoaking of oats					
	O_2			CO_2			O_2			CO_2		
	0	2	4	0	2	4	0	2	4	0	2	4
0	98	94	50	98	100	100	98	97	96	97	98	96
2	98	50	18	98	100	98	97	93	96	98	95	99
4	98	38*	10*	98	96	94	98	98	97	96	93	97

Total period of combined treatment 8 to 24 hours

Hours gas exposure	Germination of beans (%)				Germination of oats (%)			
	Dry		Presoaked 8 hrs.		Dry		Presoaked 8 hrs.	
	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2
0	100	—	90	94	97	—	92	89
0.5	96	98	84	96	93	83	99	91
1	100	94	84	96	92	89	94	90
2	90	86	52	100	90	92	90	90
4	80	94	16	96	99	90	84	90
8	10	94	0	94	70	86	62	83
16	16	100	0	64	79	85	40	84

* Seedlings in poor condition at termination of test.

This was shown further in experiments in which gas exposures of 0, 0.5, 1, 2, 4, 8, and 16 hours were followed by periods of soaking to make a total of 24 hours in water in each case, and in which presoaking times of 0, 0.5, 1, 2, 4, 8, and 16 hours were followed by corresponding periods of gas exposures to make 24-hour totals. No direct relation seems to exist between the number of hours of exposure to a gas and the resultant effect on germination.

Some of these effects where the total time in water did not exceed eight hours are shown in the first part of Table III. Here it will be seen that beans are not injured by oxygen treatment up to four hours, the same germination resulting from four hours of presoaking as from four hours of gas exposure or two hours of presoaking followed by two hours

of gas exposure. When the total time was extended to six hours injury became apparent. This injury was somewhat greater for four hours of presoaking and two hours of oxygenation than for the reverse. It appears that the seed must take up a certain amount of water and probably speed up the metabolic processes before it is susceptible to injury by oxygen. This might account for increased deleterious effect with increased presoaking time. Such differences are not obvious for the carbon dioxide treatment shown in the first part of Table III. However, when the condition of the seedlings, especially with respect to size, was considered, it was noted that in the cases of the zero and two hours of presoaking, the seedlings were more vigorous after two or four hours of the gas treatment than after shorter times in water without the gas. After four hours' presoaking, however, an additional two or four hours of carbon dioxide failed to remove all of the injurious effects of the extended soaking time (six to eight hours total).

TABLE IV

GERMINATION OF FRENCH HORTICULTURAL BEAN AND CLYDESDALE OAT SEEDS AFTER SOAKING FOR EIGHT HOURS IN WATER THROUGH WHICH VARIOUS GAS MIXTURES WERE PASSED

Seeds	Gas mixture	Germination (%) after treatment with various mixtures				
		100+0	75+25	50+50	25+75	0+100
Beans	O_2+CO_2	16	52	66	96	96
	O_2+N_2	12	10	32	52	56
	N_2+CO_2	52	76	78	100	98
Oats	O_2+CO_2	92	94	92	90	96
	O_2+N_2	82	96	97	97	95
	N_2+CO_2	97	93	97	91	91

EFFECT OF GAS MIXTURES

For these tests mixtures of gases were made in large bottles over water. A slow stream of water then forced the mixtures to bubble slowly through the water containing the seeds. A total of 48 liters of gas was used for four aeration flasks each containing 200 ml. tap water and 55 French Horticultural bean seeds or 110 Clydesdale oat seeds during an eight-hour period. At the end of this period, the seeds were placed to germinate at controlled temperatures as described above.

The effects of mixtures of oxygen and carbon dioxide, oxygen and nitrogen, and nitrogen and carbon dioxide are shown in Table IV.

Considering first the mixtures of oxygen and carbon dioxide, it will be seen that there is a progressive decrease in the injury to bean seeds as the percentage of oxygen in the mixtures decreases from 100 to 25. When the mixture contained as much as 75 per cent carbon dioxide, the subsequent

germination was not impaired by the presence of 25 per cent oxygen. That the carbon dioxide has an effect beyond that of merely excluding the oxygen from the soaking water is shown by the results of the germination after soaking with supplies of oxygen-nitrogen mixtures. With 50 per cent of the mixture nitrogen, however, there was some improvement of germi-

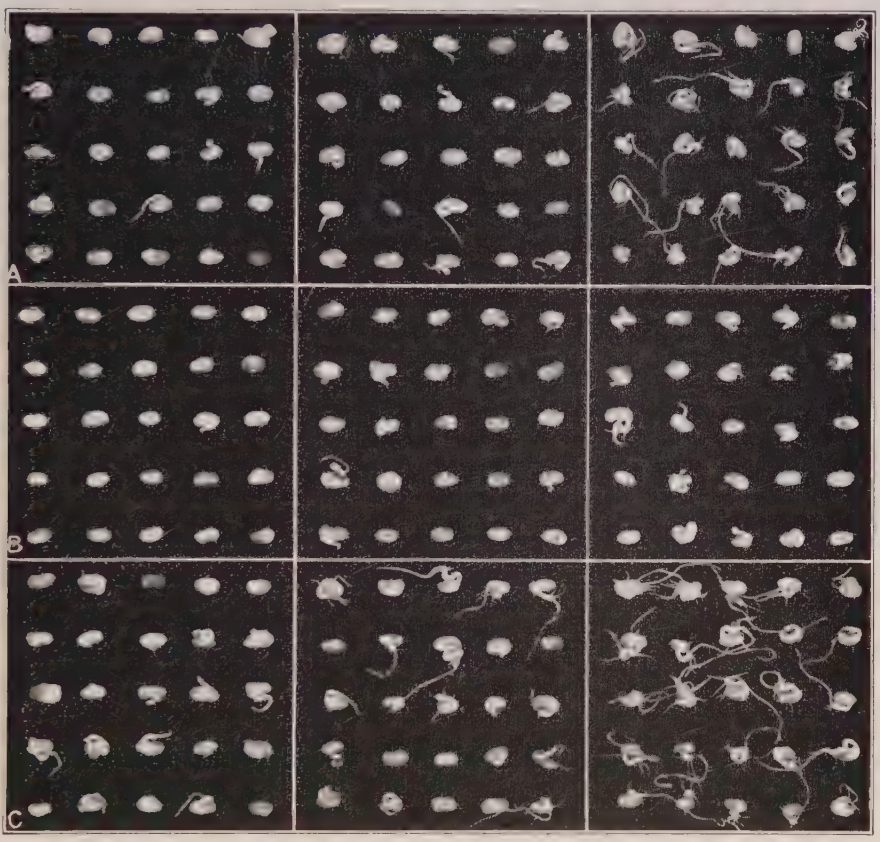


FIGURE 3. Effect on germination of gas mixtures bubbled through water in which French Horticultural beans were soaked for 8 hours. A. Oxygen+carbon dioxide; B. oxygen+nitrogen; C. nitrogen+carbon dioxide. Percentages of gases in mixtures, left to right in each group: 75+25; 50+50; 25+75.

nation over that permitted by pretreatment with 75 per cent oxygen and 25 per cent nitrogen or with a full atmosphere of oxygen. Again, as in the case of the oxygen-carbon dioxide mixtures the presence of 75 per cent nitrogen was sufficient to counteract the harmful effect of 25 per cent oxygen. One hundred per cent nitrogen supplied during the soaking process permitted only 56 per cent subsequent germination.

In the nitrogen+carbon dioxide series, it is again demonstrated that carbon dioxide has a positive stimulating effect or can prevent the deleterious effect of soaking in some way. Seeds treated with as high as 75 per cent carbon dioxide along with 25 per cent nitrogen were not injured at all, giving 100 per cent germination as compared with 52 per cent for nitrogen alone and 76 and 78 per cent for the higher proportions of 75 and 50 per cent nitrogen in the mixtures.

These effects are pictured in Figure 3. Here it can be seen that there were striking differences in the size and quality of seedlings produced under the various conditions. Thus the differences in effect are really more marked than they appear from germination percentages such as shown in Table IV. Within five hours after gas treatments had started it was possible to tell from the appearance of the bean seeds which ones were being injured. Moisture absorption in the higher oxygen mixtures was much more rapid and the color of the seeds had changed from a buff to a dark reddish brown, characteristic of oxygen injury. With increasing amounts of carbon dioxide in the mixtures the beans became less discolored and took up less water, the tendency of these seeds being to float. When 100 per cent carbon dioxide was used most of the seeds were still floating at the end of the eight-hour period. There may be a direct relation between this characteristic response and the lack of soaking injury in the presence of carbon dioxide. The difference in moisture absorption rates will be given in another section of this paper. Nitrogen effects on color of the seeds were intermediate between those of oxygen and carbon dioxide.

Once again the greater resistance of oats to soaking injury is demonstrated in Table IV. Other tests showed that presoaking for eight hours before exposing seeds of either beans or oats to the oxygen-carbon dioxide mixtures intensified the injury.

Albaum (1) also found that dilution of oxygen with nitrogen reduced the injury to oat seedlings. His work included the soaking of oat seeds for 20 hours in water through which oxygen-nitrogen mixtures containing 0, 2.5, 5, 10, 20, 50, and 100 per cent oxygen were bubbled. After exposure to 100 per cent oxygen the germinating seeds failed to develop normal coleoptiles, their length being inhibited to the extent of 85 per cent. Other gases or mixtures were not used by Albaum.

EXCISED EMBRYOS VS. INTACT SEEDS

Excised embryos as well as intact seeds of corn were exposed to air and to oxygen in the manner described above for a period of 24 hours, after which they were planted in soil in the greenhouse. The results may be seen in Figure 4. The naked embryos were injured by both air and oxygen (Fig. 4, A and B), but the greater injury was by oxygen. Seedlings from intact seeds were normal after both air and oxygen treatment, but germination was reduced by the latter (Fig. 4 C and D).

FIGURE 4. Effect of oxygen on the germination of corn embryos and intact seeds soaked in water through which air or oxygen was bubbled for 24 hours. Excised embryos: A. air, B. oxygen. Intact seeds: C. air, D. oxygen.

Excised embryos of the sunflower (*Helianthus*) were also injured by oxygen. The peculiar constrictions in the hypocotyls of the oxygen-injured embryos shown in Figure 5 are typical. In this case a third set of embryos was soaked in water through which oxygen and carbon dioxide were bubbled simultaneously for 24 hours. Comparison of these effects with those of oxygen alone, and air may be seen in Figure 5 A, B, and C. It will be noted that the presence of carbon dioxide seemed to reduce the harmful

FIGURE 5. Growth on moist filter paper of excised embryos of sunflower soaked previously in water through which air (A), oxygen (B), or both oxygen and carbon dioxide (C) was bubbled for 24 hours.

effect of the oxygen. We have also had an indication that carbon dioxide in the soaking solution reduces the injury of oxygen to bean seeds.

So far we have seen that both excised embryos and intact seeds are sensitive to oxygen. Seeds of cocklebur (*Xanthium*) exhibit a different response. In the bur of this species there are two seeds, the upper borne near the apical end of the bur and the lower lying near the base of the bur, which are very different from each other in their physiological behavior.

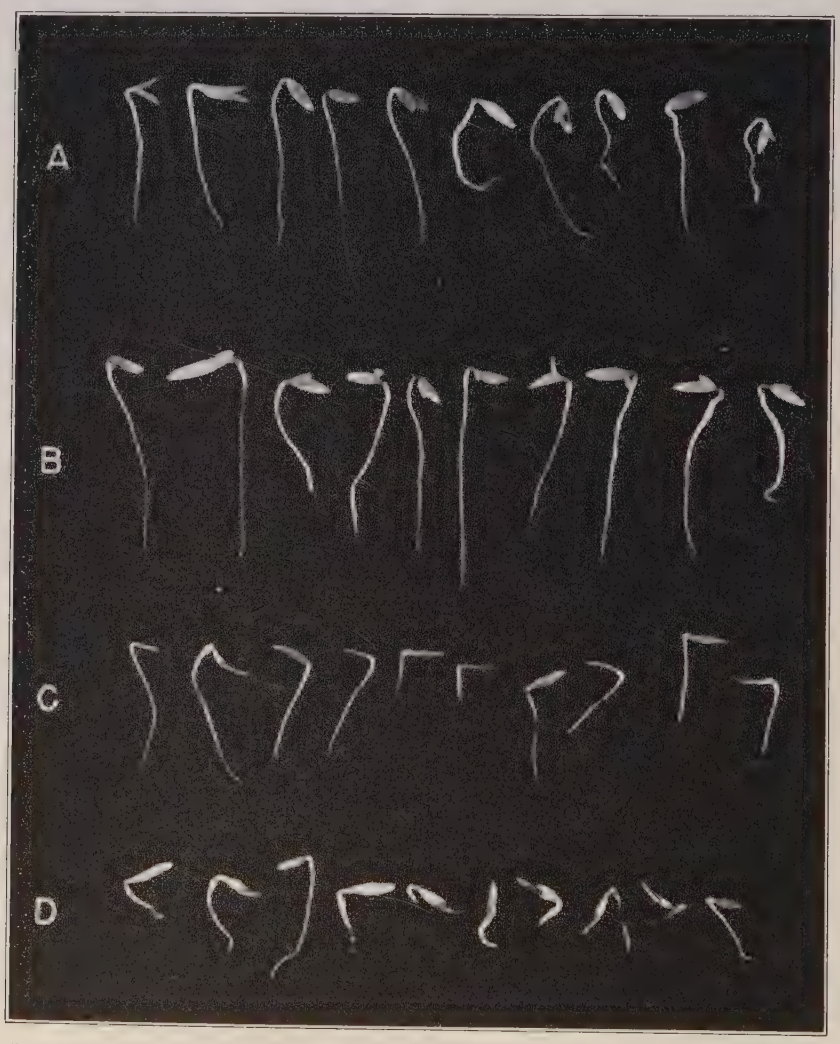


FIGURE 6. Lower seeds of *Xanthium*. Excised embryos and intact seeds soaked in water through which air or oxygen was bubbled for 24 hours, after which they were placed on moist filter paper at 30° C. for two days. Excised embryos: A. air; B. oxygen. Intact seeds: C. air, D. oxygen.

Typically, the lower seed germinates readily, while the upper one displays a type of dormancy which may be broken by exposure to extra oxygen pressures supplied in the atmosphere above the seeds which are on a moist substratum (Thornton 12). Because of this characteristic, these seeds were included in the oxygen studies here reported. There was no apparent injury to the excised embryos of either the upper or the lower seeds when the exposure to air or oxygen dissolved in water was not longer than 24 hours. However, when the time of exposure was 48 hours, embryos exposed to oxygen showed evidence of greater injury than those exposed to air. These embryos were kept on moist filter paper at 30° C. for three or four days after treatment. During this period they elongated very rapidly so that some of them were as much as 15 cm. long. Browning of some of the root tips was seen in all cultures and may have been associated with the length of time the seedling was on moist filter paper, and yet many seedlings which had been exposed to air remained firm and vigorous for as long as 6 days after treatment.

At the same time that excised embryos were treated, intact upper and lower seeds of the cocklebur were also soaked in water through which air or oxygen was bubbled. In Figure 6 it will be seen that, while the excised embryos of the lower seeds were apparently not injured at all by 24 hours of exposure to air or oxygen (A and B), the embryos were injured inside the intact seed coat, as shown by the delayed germination followed almost immediately by the rotting of the roots (Fig. 6 C and D). This was not so evident for embryos of the upper intact seeds for few germinations were secured from them. The explanation for this behavior is not apparent at this time. Since excised embryos of corn and sunflower have been shown to be very sensitive to oxygen injury, obviously we cannot conclude that all seed coats increase the harmful effect of oxygen. Cocklebur may prove to be unique in this regard. Also preliminary tests have shown that the incubation temperature after treatment may affect the extent of the injury. At the more favorable germination temperature of 35° C., for example, seedlings from intact cocklebur seeds soaked in water supplied with oxygen were comparable in growth to those supplied with air.

CAUSES OF HARMFUL EFFECTS OF SOAKING

Among the explanations advanced for the deleterious effects of soaking bean seeds are those of Kidd and West (10) who stated that there were three plausible hypotheses: 1. disorganized metabolism resulting from deficiency in oxygen supply and accumulation of carbon dioxide; 2. leaching out of essential soluble food reserves; and 3. a combination of 1 and 2. They did not think it was lack of oxygen and they were unable to correlate leaching effects with temperature effects, since exosmosis was greatly increased at 30° C., over that at 20° to 25° C., and yet the injury was less

at the higher temperature. All of the harmful effect of soaking has been attributed to bacterial action by Tilford *et al.* (14) and Barton (4). Eyster (7), while recognizing the reduction in germination of beans soaked in contaminated water, maintained that an insufficient oxygen supply and a loss of essential cell constituents were also responsible.

The experimental results described in the present paper show clearly that any disorganization of the metabolism of the seed is not due either to a deficiency in oxygen supply or to an accumulation of carbon dioxide, but rather the reverse might apply. Data are also at hand indicating that bacterial action is not the sole cause of deterioration. That materials do leach out of the seeds during the soaking process is evident. Furthermore the carbon dioxide treatment decreased the exosmosis and oxygen increased it as measured by the color of the solutions at the end of the 24-hour soaking period.

Also, experiments have shown that leached substances are capable of inhibiting the growth of wheat roots in solution. Increasing concentrations of the soaking solutions caused greater reductions in growth. There was some evidence that more inhibiting substances leached out of the seeds in the presence of air and oxygen than in the presence of nitrogen, carbon dioxide, or in non-aerated water. However, since water extracts of the dry or soaked seeds inhibit the growth of wheat roots (5), it may be the quantity rather than the nature of the leached materials that is important. Thus we may be measuring a leaching of vital chemicals from the seeds which process may contribute to the harmful effect of soaking and which may be accelerated by the presence of certain gases. In this connection it may be said that replacement of water by a complete nutrient solution failed to prevent soaking injury.

Since carbon dioxide has an acidifying effect on the solution through which it is passed, this phase of the subject was investigated. Measurements of the pH of the water in which the seeds had soaked for 24 hours, as well as adjustments of the hydrogen ion concentration by adding 0.2 M dibasic sodium phosphate and 0.1 M citric acid to distilled water to give a pH range of 8.2 to 3.1 failed to show any direct relationship between acidity and soaking injury.

It was thought that the rate of moisture absorption of beans soaking in the presence of different gases might be related to germination injury. An experiment was planned in which duplicate lots of French Horticultural beans were weighed after 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 hours in non-aerated water and in water supplied with air, oxygen, nitrogen or carbon dioxide. The air-dry weights of 130 lots of 50 seeds each were recorded before placing them in water. A uniform procedure was followed for removing all surface moisture from the seeds before the sample was placed in a weighing bottle. Since there was close agreement between dup-

licate samples from the same soaking period, the results are given as averages of the duplicates (Table V).

There was a striking reduction in the rate of moisture absorption under treatment with carbon dioxide as compared with all other conditions after the two-hour period. Also there was a tendency for greater absorption with oxygen supplied than with air or nitrogen or in the non-aerated lots, in spite of the apparently greater loss of materials by leaching. Air induced greater absorption than nitrogen. Of course it is not definitely proved that the extra water absorption is in itself detrimental. However it

TABLE V

MOISTURE ABSORBED BY FRENCH HORTICULTURAL BEANS WHEN SOAKED IN WATER SUPPLIED WITH DIFFERENT GASES

Hrs. of soaking	Per cent moisture absorbed when supplied with				
	No gas	Air	Oxygen	Nitrogen	Carbon dioxide
1	10.5	17.1	14.3	14.3	11.8
2	28.1	41.8	36.9	31.0	16.3
4	71.6	78.6	77.0	78.6	31.8
6	86.3	99.9	105.5	94.9	43.4
8	99.3	106.5	118.8	103.5	45.1
10	106.5	112.9	120.6	105.6	62.1
12	108.3	115.7	120.7	111.0	68.1
14	109.2	117.6	121.6	110.5	74.3
16	111.6	118.8	123.6	111.5	81.9
18	111.8	119.8	125.8	111.4	83.3
20	112.9	121.6	124.5	110.7	87.7
22	113.8	120.9	125.9	113.6	91.4
24	112.9	120.9	126.3	114.5	96.1

is noteworthy that dry bean seeds of the same lot when rolled in moist paper towels for normal germination have absorbed only 83.2 per cent moisture when germination begins after 28 hours. The moisture percentages given here were calculated on the basis of the air-dry weights of the seeds. On the same basis these air-dry seeds contained 6.8 per cent moisture as determined by drying in a vacuum oven at 71° C.

The presence of carbon dioxide in the water causes bean seeds to float. From this behavior and the appearance of the seeds, it seems that this gas penetrates the seed coats much more readily than does air, nitrogen, or oxygen and thus forms a gas layer between the seed coats and the embryo. This layer not only causes the seeds to float, but may reduce the rate of water absorption by placing a barrier between the source of supply and the embryo. Solutions of surface-active agents did not prevent the floating of bean seeds in the presence of carbon dioxide. In line with these results, a lessening of the deleterious effect of soaking seeds of *Pisum sativum* and Tendergreen variety of beans as compared with Golden Cluster Wax

beans (Table I) is thought to be due to the greater resistance to moisture absorption of the seed coats of the two former.

It is of interest to note that water absorption by roots of wheat, corn, and rice plants growing in water cultures is reduced 14 to 50 per cent by bubbling carbon dioxide through the solutions (6). This effect is described as toxic since the growth of the plants was adversely affected by reduced absorption.

From careful enzyme assays and studies of nitrogen content of different organs, Albaum, Donnelly, and Korkes (2) concluded that the failure of oat grains to develop normal coleoptiles after exposure of the seeds to oxygen may be due to the failure of the protein in the endosperm to break down, thus preventing nitrogen transport and the development of the enzyme systems. Also there was indication that amino-nitrogen failed to move from the endosperm to the embryo.

SUMMARY

It has been shown that soaking injury to seeds of *Phaseolous vulgaris*, *P. lunatus*, *Pisum sativum*, *Vicia faba*, *Helianthus annuus*, *Avena sativa*, *Hordeum vulgare*, *Triticum aestivum*, and *Zea mays* is enhanced by passing oxygen through the water in which the seeds are soaked. Passing air, nitrogen, or hydrogen in like manner reduced the harmful effect but did not permit normal germination. Carbon dioxide treatment prevented the deleterious effects resulting from soaking several different varieties of beans, but caused decreased germination of some grains.

An excess of water in the germination medium reduced the germination of beans and oats after soaking treatments.

Injury to oxygenated beans was apparent after six hours of soaking and became more pronounced after 12 or 24 hours. Presoaking in water through which no gases were passed increased the oxygen injury. The effect of the gases used could not be entirely separated from the effect of the water itself in the soaking process.

In water supplied with mixtures of different proportions of oxygen and carbon dioxide, bean seeds showed progressive injury with increased amounts of oxygen. When the mixture contained as much as 75 per cent carbon dioxide, the subsequent germination was not impaired by the presence of 25 per cent oxygen. The use of oxygen-nitrogen and nitrogen-carbon dioxide mixtures demonstrated that the favorable effect of the carbon dioxide was not due solely to the exclusion of oxygen.

Both excised embryos and intact seeds of corn and sunflower were adversely affected by oxygen supplied during soaking, but the embryos were more sensitive. Excised embryos of *Xanthium*, on the other hand, were not easily injured by oxygen but were more sensitive while still enclosed in the seed. This latter effect was not noted in any other species.

A pronounced reduction in the water absorption rate of bean seeds soaked in the presence of carbon dioxide indicated a direct relationship between this behavior and injury from soaking. Also there was a tendency for absorption far beyond the point needed for germination when oxygen was supplied during the soaking process. Air induced greater absorption than nitrogen. It appears from the data, however, that there are certain gas effects which are independent of the effects of moisture absorption.

LITERATURE CITED

1. ALBAUM, HARRY G. The growth of oat coleoptiles after seed exposure to different oxygen concentrations. Biol. Bull. **79**: 360–361. 1940.
2. ALBAUM, HARRY G., JOHN DONNELLY, and SEYMOUR KORKES. The growth and metabolism of oat seedlings after seed exposure to oxygen. Amer. Jour. Bot. **29**: 388–395. 1942.
3. BAILEY, WILLIAM MARSHALL. Structural and metabolic after-effects of soaking seeds of *Phaseolus*. Bot. Gaz. **94**: 688–713. 1933.
4. BARTON, ARTHUR W. The effect of long-continued soaking on seed germination. Trans. Kansas Acad. Sci. **32**: 37. 1929.
5. BARTON, LELA V., and MARIE L. SOLT. Growth inhibitors in seeds. Contrib. Boyce Thompson Inst. **15**: 259–278. 1948.
6. CHANG, H. T., and W. E. LOOMIS. Effect of carbon dioxide on absorption of water and nutrients by roots. Plant Physiol. **20**: 221–232. 1945.
7. EYSTER, H. CLYDE. The cause of decreased germination of bean seeds soaked in water. Amer. Jour. Bot. **27**: 652–659. 1940.
8. KIDD, FRANKLIN. The controlling influence of carbon dioxide in the maturation, dormancy, and germination of seeds. Part II. Proc. Roy. Soc. [Lond.] B. **87**: 609–625. 1914.
9. KIDD, FRANKLIN, and CYRIL WEST. Physiological pre-determination: The influence of the physiological condition of the seed upon the course of subsequent growth and upon the yield. I. The effects of soaking seeds in water. Ann. App. Biol. **5**: 1–10. 1918.
10. ———. The influence of temperature on the soaking of seeds. New Phytol. **18**: 35–39. 1919.
11. KISSER, J., und J. POSSNIG. Untersuchungen über den Einfluss gehemmter und geförderter Sauerstoffatmung auf Samenkeimung und Keimlingswachstum. Beitr. Biol. Pflanzen **20**: 77–104. 1933.
12. THORNTON, NORWOOD C. Factors influencing germination and development of dormancy in cocklebur seeds. Contrib. Boyce Thompson Inst. **7**: 477–496. 1935.
13. ———. Carbon dioxide storage. IX. Germination of lettuce seeds at high temperatures in both light and darkness. Contrib. Boyce Thompson Inst. **8**: 25–40. 1936.
14. TILFORD, PAUL, C. F. ABEL, and R. P. HIBBARD. An injurious factor affecting the seeds of *Phaseolus vulgaris* soaked in water. Papers Michigan Acad. Sci., Arts & Letters **4**: 345–356. 1924.

TOLERANCE OF SULFATE-REDUCING BACTERIA TO HYDROGEN SULFIDE

LAWRENCE P. MILLER

When bacteria are grown under conditions in which nutrients are not replenished and the products of bacterial metabolism are not removed the growth attainable in a given culture is naturally limited. When the essential nutrients are in excess, development will cease either because of an accumulation of the main product of the bacterial action or because of the presence of deleterious quantities of the products of side reactions. With sulfate-reducing bacteria, *Desulfovibrio desulfuricans* (Beij.) K. and v. N., which produce high concentrations of hydrogen sulfide, it is logical to suspect hydrogen sulfide as the limiting factor since this is known to be highly toxic to most forms of life.

In previous experiments concerned with a possible relation of sulfate-reducing bacteria to the commercial sulfur deposits, it was found that through suitable changes in the media and cultural conditions these bacteria could produce much higher concentrations of hydrogen sulfide than had previously been thought possible (3). Both non-marine and marine forms produced up to 2500 mg. per liter of the sulfide in contrast to the previously reported maxima of 794 and 1448 mg. per liter for these forms (1). The question remains whether this maximum of 2500 mg. per liter is determined by the sensitivity of the bacteria to hydrogen sulfide or by some other factor or factors. It also is of interest to determine whether individual cultures, which produce much less than the maximum possible as a result of inoculation from weak cultures or some other unfavorable conditions, fail to do so because of an increased sensitivity of the bacteria to hydrogen sulfide. Experiments bearing on these points are reported in the present paper. Data are also presented on the effect of age of the culture used as inoculum on the rate of growth of the subsequent culture and on the survival time of cultures containing high concentrations of hydrogen sulfide when maintained at 30° C. or 0.5° C. While these results show that removal by one means or another of the hydrogen sulfide formed in a culture permits a larger total amount of sulfide to be formed, nevertheless they also demonstrate the rather remarkable tolerance to hydrogen sulfide exhibited by this organism.

MATERIALS AND METHODS

The source of the bacteria was the same as described in a previous paper (3). Since no differences between strains were established in the tests reported, specific strains are not referred to.

Copyright, 1950, by Boyce Thompson Institute for Plant Research, Inc.

The procedures followed for culturing the bacteria and for determining the quantity of hydrogen sulfide formed were also the same as reported in the previous paper with the exception that culture media were not freshly heated and cooled each day. For the most part the experiments were carried out with a medium previously designated as No. 220. This contains the following ingredients in grams per liter: NH_4Cl, 1.00; $MgSO_4 \cdot 7H_2O$, 2.00; Na_2SO_4, 18.25; K_2HPO_4, 0.50; $CaCl_2 \cdot 2H_2O$, 0.10; sodium lactate, 21.0; NaCl, 10.0; $CaCO_3$, 1.0; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, trace. In experiments in which relatively large amounts of ferrous sulfate were added to the medium a special medium was used which is described in the appropriate section below. In some of the tests in which cadmium carbonate was added to remove hydrogen sulfide as it was formed enriched media were used. These were based on No. 220 but contained more sodium sulfate and sodium lactate and thus furnished enough necessary nutrients to permit the formation of larger quantities of hydrogen sulfide.

RESULTS

LACK OF SENSITIVITY OF CULTURES TO ADDITIONAL HYDROGEN SULFIDE

In order to determine whether cultures producing relatively low concentrations of hydrogen sulfide are sensitive to the sulfide, a number of tests were carried out in which rather large amounts of extra hydrogen sulfide were added and the effect upon growth following transfer studied. The material used for these experiments consisted of a number of crude cultures, both marine and non-marine in origin, which had been held at 30° C. for 20 to 27 days and which had consequently reached their maxima as to hydrogen sulfide production. Two 1-ml. aliquots were withdrawn as inocula for two new cultures, another portion was used for the quantitative determination of the hydrogen sulfide content, and a third portion was exposed to hydrogen sulfide gas from a Kipp generator in a closed system. After varying times of exposure the hydrogen sulfide was again determined, the content now being the sum of the hydrogen sulfide originally formed by bacterial action and that obtained from the Kipp generator. Two more new cultures were started with inoculum after the exposure to the additional hydrogen sulfide. In Table I are listed the original hydrogen sulfide contents of the various cultures, length of exposure to additional hydrogen sulfide and the concentration of hydrogen sulfide at the end of the exposure period, and the hydrogen sulfide content reached in new cultures from inocula obtanied before and after the further addition of hydrogen sulfide. It is clear that exposure to concentrations up to 2700 mg. per liter for time periods from one-half to 22 hours had no deleterious effect on growth following subsequent transfer as determined by the concentration of hydrogen sulfide reached in the new cultures. While these tests offer no direct evidence as to whether development ceased primarily because of

TABLE I

TOLERANCE OF CULTURES OF SULFATE-REDUCING BACTERIA YIELDING LOW CONCENTRATIONS OF HYDROGEN SULFIDE TO RELATIVELY LARGE AMOUNTS OF ADDITIONAL HYDROGEN SULFIDE

H_2S formed in original culture	Content after addition of more H_2S	Time of exposure to additional H_2S, hrs.	H_2S formed upon transfer, mg./l.	
			From culture with additional H_2S	Without added H_2S
1223	2746	0.5	1168, 1447	1344, 1311
406	2616	0.5	1285, 1232	1184, 1155
1033	2146	0.5	1357, 1286	1312
1319	2696	1.0	1209, 1281	1453, 1236
1345	2357	18.0	1247, 1349	1222, 1341
395	1646	4.5	399, 404	388, 445
1503	2333	1.5	1716, 1748	1738, 1757
450	2067	22.0	855, 442	330, 330
368	2114	22.0	211, 265	1068, 1104

the hydrogen sulfide content reached by the cultures, they do show that much higher concentrations could be tolerated by the bacteria of these cultures at least for periods up to 22 hours.

EFFECT OF REMOVAL BY ZINC ACETATE IN THUNBERG TUBES OF SOME OF THE HYDROGEN SULFIDE FORMED

The experiments described in the previous section show that the bacteria in cultures yielding relatively low concentrations of hydrogen sulfide are not sensitive to considerably larger amounts of hydrogen sulfide. It is of interest to determine whether removal of the hydrogen sulfide formed will result in further development and formation of additional amounts of the sulfide. Thunberg tubes were arranged with a solution of zinc acetate in the upper arm to absorb the hydrogen sulfide given off from the culture medium in the main body of the tube. Experiments with cultures free of contaminating organisms in such tubes showed that removal of even a part of the hydrogen sulfide resulted in an increased total production. Similar results were obtained whether the cultures were maintained in the tubes in an atmosphere of nitrogen or under vacuum.

In one test part of a culture in which 1503 mg. per liter of hydrogen sulfide had developed was transferred to a Thunberg tube containing zinc acetate in the upper arm. After 88 more hours the total quantity of hydrogen sulfide present in the Thunberg tube was 1942 mg. per liter while that in an undisturbed replicate culture was 1636. In another experiment a culture containing 1323 mg. per liter reached a total of 2206 mg. per liter in a Thunberg tube while a replicate culture in which the hydrogen sulfide was not removed produced only 1503 mg. per liter. In two more such tests concentrations equivalent to 1692 and 1923 mg. per liter were formed in the Thunberg tubes while the regular cultures produced only 1293 and 1780 mg. per liter. These experiments thus indicate that the presence of hydrogen

sulfide in the cultures is a deterrent to the further production of hydrogen sulfide.

REMOVAL OF THE HYDROGEN SULFIDE BY CONSTITUENTS OF THE NUTRIENT MEDIUM

It would seem that an ideal way to study the effect of hydrogen sulfide on the rate of production and on the total amount formed would be to arrange experimental conditions so that the hydrogen sulfide is removed as it is being formed. Two general methods suggest themselves, one involving passing an inert gas through the culture medium to remove the hydrogen sulfide, and the other the use of a constituent in the nutrient medium which would precipitate the sulfide produced. The first method presents some difficulties in regard to maintaining pure cultures and also in introducing mechanical agitation of the cultures. For the use of the second method it is necessary to have a substance which is in itself not harmful to the development of the bacteria and which on reaction with hydrogen sulfide does not produce a product inimical to their growth. Experiments have shown that the bacteria will develop readily in media containing ferrous sulfate or cadmium carbonate, which precipitates the sulfide produced as ferrous and cadmium sulfides, respectively. In the presence of either of these materials the total amount of sulfide produced was usually greater than in their absence, thus indicating that under the usual cultural conditions the concentration of hydrogen sulfide produced retards further development unless one would assume that the ferrous and cadmium salts remove other deleterious substances as well as hydrogen sulfide. Rates of development were not increased in the presence of either ferrous sulfate or cadmium carbonate but rather somewhat retarded.

Use of ferrous sulfate. Ferrous sulfate might seem to be an excellent substance to use for the removal of sulfide as formed since the sulfate in turn would be reduced by bacterial action. The formation of ferrous sulfide in nature as a result of the activities of the sulfate reducers is commonly observed as manifested by the black muds often found where this organism is active. The use of ferrous sulfate entailed some difficulties, however, since the ferrous salt is quite easily oxidized in air to the ferric salt and furthermore its addition to the medium decreased the pH to a value too low for the development of the bacteria. The presence of ferric ions is undesirable since they are reduced by hydrogen sulfide with oxidation of the sulfide to elemental sulfur. Nevertheless some successful experiments were carried out in which a large quantity of ferrous sulfate was added to the nutrient medium. The pH was corrected by using relatively large amounts of calcium carbonate. Under such conditions growth occurred readily. For the determination of the sulfide produced it was necessary to add an acid, such as acetic or preferably hydrochloric, drive off the hydrogen sulfide

released by passing a stream of N through the culture, absorb the hydrogen sulfide in a solution of zinc acetate, and titrate with iodine after forming hydrogen sulfide from the zinc sulfide with the use of hydrochloric acid. Attempts to determine the sulfide by titrating the cultures directly in a 500 ml. bottle after adding acid often led to high results—possibly as a result of the addition of iodine before all of the excess calcium carbonate had been dissolved.

A medium containing the following ingredients, expressed in grams per liter, supported the growth of the bacteria with the formation of larger quantities of total sulfide than had been obtained previously: $FeSO_4 \cdot 7H_2O$, 60; NH_4Cl, 1.0; $MgSO_4 \cdot 7H_2O$, 2.00; K_2HPO_4, 0.50; NaCl, 10.0; sodium lactate, 56.0; peptone, 10.0; $CaCO_3$, 75. The same medium without peptone was also used. In one test the sulfide produced in this medium without peptone was equivalent to 2697 mg. per liter of hydrogen sulfide while the maximum produced in four replicate cultures in medium 220 was 1753 mg. per liter. Similarly, when peptone was present the equivalent of 2359 and 2087 mg. of hydrogen sulfide per liter was formed while the control in 220 produced only 1668 mg. per liter. In another test 1556 and 1710 mg. per liter were produced as compared to 1413 mg. per liter. Cultures in these media in which all the sulfide wh ch has been formed is present as ferrous sulfide, as indicated by the lack of odor of hydrogen sulfide, are still viable although they are about one and one-half years old.

In the interval since these cultures were started it had been found that cadmium carbonate is suitable for the removal of the sulfide as formed. The question of oxidation in air and too high acidity does not arise and the cultures can be titrated in bottles directly after the addition of acetic acid or preferably hydrochloric acid. It is necessary to allow sufficient time for the solution of the cadmium sulfide, especially with older cultures. Results are presented below.

Use of cadmium carbonate. Since solutions of cadmium salts are often used for the absorption of hydrogen sulfide and its subsequent determination after dissolving the cadmium sulfide in acid, the possibility of using cadmium as a means of removing hydrogen sulfide as it was being formed suggested itself. Naturally the bacteria would not be expected to develop in the presence of an appreciable concentration of cadmium ions. It is necessary, therefore, to use a salt of limited solubility which does not contain a deleterious anion which would be released on reaction with hydrogen sulfide. Cadmium carbonate seemed to fulfill this requirement and tests showed that the bacteria developed readily in its presence. The relative solubilities of the carbonate and sulfide are such that the former reacts with the hydrogen sulfide as it is being produced. Cultures containing much sulfide were free of the odor of hydrogen sulfide. Extensive rate studies were not carried out but it is clear from observations of the cultures, as

compared to cultures in media without cadmium carbonate, that the presence of the latter lengthened the lag period somewhat. However, vigorous development occurred and the total amounts of sulfide formed were larger than the quantity of hydrogen sulfide produced in the control cultures. The medium used was the regular 220 medium or a modification of it containing additional sulfate and lactate to furnish sufficient nutrients to permit larger quantities of sulfide to be produced. Two grams of cadmium carbonate were added to each culture vessel. This quantity, if all

TABLE II

MAXIMUM QUANTITIES OF SULFIDE, EXPRESSED AS MILLIGRAMS OF HYDROGEN SULFIDE PER LITER, PRODUCED BY SULFATE-REDUCING BACTERIA WHEN CADMIUM CARBONATE WAS ADDED TO THE MEDIUM

With $CdCO_3$	Control	With $CdCO_3$	Control
2808	2401	2694	1978
2666	1998	2668	2086
2896	2034	2153	1915
2880	2171	3339	2208
2778	1908	2923	2085
2759	1751	3239	2334

converted to the sulfide, is equivalent to about 6,000 mg. per liter of hydrogen sulfide. Results are given in Table II. In every case considerably larger amounts of total sulfide were formed when cadmium carbonate was present in the medium.

SURVIVAL OF BACTERIA IN CULTURES CONTAINING RELATIVELY HIGH CONCENTRATIONS OF HYDROGEN SULFIDE

Previous workers have found that sulfate reducers can be kept alive in artificial media for long periods of time. Survival for a year or longer has been reported (2). Since in the present experiments considerably higher concentrations of hydrogen sulfide than had previously been obtained were involved, it seemed of interest to study the effect of these higher concentrations on survival. For a consideration of the effects of long exposure to high concentrations of hydrogen sulfide, two types of data are available. For cultures less than 50 days old rate of growth, as measured by hydrogen sulfide formation, was determined and thus an indication of vigor is also given by the data. Survival for longer periods when kept at either 30° or 0.5° C. was determined on the basis of whether or not development took place upon transfer.

Effect of age of culture on rate of growth on transfer. In connection with previous experiments designed to determine the maximum concentrations of hydrogen sulfide that the bacteria could produce, it was found that more vigorous cultures forming higher concentrations of hydrogen sulfide more

rapidly resulted when intervals between transfers were relatively short, that is, a week or less. Much information was obtained on the effect of the age of the culture from which the inoculum was taken on the rate of growth of a new culture, as measured by the rate of formation of hydrogen sulfide. Results of 234 such tests are summarized in Figure 1. It is seen that most rapid development took place when the culture from which the inoculum was taken was about two days old. A more or less regular decline in vigor

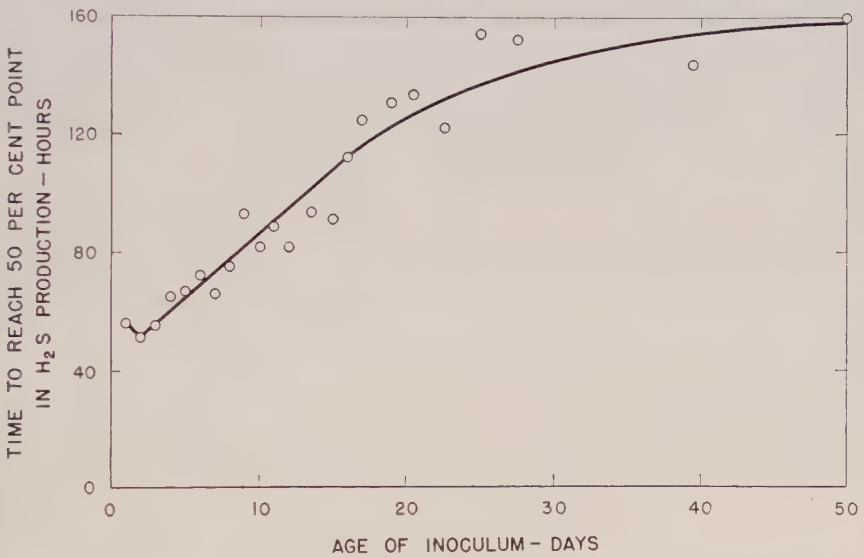


FIGURE 1. Relation between age of inoculum and the rate of hydrogen sulfide formation.

was evident as the age of the inoculum increased from about 3 days to 20 days. There is no evidence for any further decline from about 21 days to 50 days. The results thus show a survival in good condition for at least 50 days. Data for longer periods are presented below.

Survival at 30° and at 0.5° C. For cultures older than 50 days some data are available as to whether or not growth occurred when transfers were made. Rate studies were not carried out with these older cultures. Data for cultures stored at 30° and at 0.5° C. are presented in Table III. It is seen that at 30° only 63 per cent of the cultures from 50 to 100 days old were viable and as age increased up to 300 days the percentage of viable cultures decreased further to about 15 per cent. One of two cultures kept over 300 days grew upon transfer.

As would be expected the bacteria remained viable for longer periods at 0.5° C. Cultures held up to 100 days were all viable and the percentage

of cultures remaining viable was decreased only to about 50 per cent for 16 cultures held from 301 to 343 days at this temperature. A basic difference between the cultures at 30° and at 0.5° is that no growth, as measured by hydrogen sulfide production, took place at the lower temperature. The procedure used in these viability studies was to hold the cultures a number of days at 30° for growth to occur before storing them at 0.5° C. Therefore, at 30° the quantity of hydrogen sulfide reached was the maximum possible for that particular culture, while the hydrogen sulfide content of the cultures stored at 0.5° was dependent upon the degree of development at 30° before transfer to 0.5°. The length of time the cultures were held

TABLE III

SURVIVAL OF CULTURES OF SULFATE REDUCERS KEPT FOR COMPARATIVELY LONG PERIODS AT 30° AND AT 0.5° C.

30°			0.5°		
Age, days	No.	Percentage viable	Age, days	No.	Percentage viable
50–100	16	63	50–100	12	100
101–200	13	23	101–200	8	50
201–300	15	13	201–300	9	56
301–319	2	50	301–343	16	56

at 30° was not the same for all the cultures included in this study since the time necessary to reach high concentrations of hydrogen sulfide varied with the age of the parent culture. In general, the extent of exposure to hydrogen sulfide was essentially the same in the 0.5° as in the 30° series.

For each culture contributing to the averages given in Table III the age and the hydrogen sulfide content when transferred were known. An examination of these data, especially from the 0.5° series, indicated that long exposure to high concentrations of hydrogen sulfide did not seem to be the primary reason for only about 50 per cent of the cultures being viable when held for over 100 days. Often among cultures of approximately the same age, those containing high concentrations of hydrogen sulfide remained viable while others containing less sulfide failed to grow upon transfer. The fact that more hydrogen sulfide was produced indicated greater vigor in the first instance, and apparently factors associated with this vigor were more important in determining survival time than any injurious effect of the accumulated hydrogen sulfide. The effect of contact with hydrogen sulfide is brought out clearly when the data are arranged in order with respect to degree of exposure. The degree of exposure may be given by an exposure index which can be obtained by multiplying the number of days the cultures were held by the concentration of hydrogen sulfide present in grams per liter. When the data for the 0.5° series were arranged

in descending order with respect to the magnitude of this index, it was apparent that survival was quite independent of the concentration of hydrogen sulfide. Thus, of the 15 cultures with the highest indices (698–266) 80 per cent grew upon transfer. The next 15 cultures with indices varying from 263 to 134 had a survival rate of 73 per cent, and the 15 cultures with the lowest indices, 132 to 36, manifested a survival rate of 60 per cent. There is thus no evidence that the survival time was determined by sensitivity to hydrogen sulfide.

Since there were definite decreases in survival rate in the 30° series with time, an analysis such as that carried out above would show decreased survival with the higher indices. Such would also be the case, of course, if the 0.5° series had been carried to the time when increasing numbers of cultures had failed to grow on transfer. Such data would not prove that the loss in viability was the result of sensitivity to hydrogen sulfide. All that can be stated with the information at hand is that the bacteria are able to withstand high concentrations of hydrogen sulfide for long periods of time and that reasons for loss in viability are not apparent but that sensitivity to long exposure to hydrogen sulfide does not seem to be the primary factor.

DISCUSSION

Although there are many organisms which may form small amounts of hydrogen sulfide or which can live in the presence of low concentrations of sulfide, the sulfate-reducing organism is unique in being able to withstand extremely high concentrations. Because of the general toxicity of hydrogen sulfide to various forms of life, workers interested in the sulfate reducers have been tempted to assume that the hydrogen sulfide they produce is inimical in one way or another to their development. Thus the maxima suggested by Baars 20 years ago, namely 794 mg. per liter for non-marine forms and 1448 mg. per liter for marine forms (1), were long accepted until recent work showed that these maxima could be easily exceeded through relatively minor changes in the nutrient media (3). In fact, with the improved media, these maxima were occasionally exceeded during the first growth period in a culture vessel, after inoculation with samples of soil. Furthermore, these experiments also showed that production of high concentrations of hydrogen sulfide was not dependent upon relatively slow growth but that on the contrary rapid development of the bacteria led to the highest concentrations of the sulfide observed.

Since this organism will not grow in the absence of sulfate and since the reduction of sulfate to hydrogen sulfide is a necessary part of its existence, it would not be expected that the quantities of hydrogen sulfide ordinarily produced within the cells would have any particularly harmful effect. On the other hand, accumulation of large quantities of the end product of its

metabolism, like an accumulation of the end products of the activities of other species of bacteria, even when such products in themselves are not especially toxic, would be expected ultimately to prevent further development. The experiments reported in the present paper have shown that when some or all of the hydrogen sulfide produced is removed, a greater total quantity of sulfide is formed; this would indicate clearly that the presence of large quantities of hydrogen sulfide tends to retard the production of additional sulfide. However, the bacteria were tolerant to high concentrations of hydrogen sulfide and survival in old cultures did not seem to be primarily associated with the concentration of hydrogen sulfide.

Removal of an end product of bacterial metabolism as it is being produced, regardless of whether the substance could be considered particularly toxic, would be expected to increase the total quantity of end product formed and probably also the rate of formation. In the present experiments when the sulfide was removed in one way or another the total quantity formed was increased but not the rate of production. Possibly the methods used involved conditions in themselves somewhat deleterious to the bacteria.

It would be of interest to be able to determine the quantity of sulfide present within the bacteria when growing in media in which high concentrations are found. Quite likely concentrations within the bacterial cells differ markedly from those outside.

SUMMARY

Bacteria in individual cultures of the sulfate-reducing organism, *Desulfovibrio desulfuricans* (Beij.) K. and v. N., which for one reason or another do not produce as high concentrations of hydrogen sulfide as are ordinarily possible, are not especially sensitive to considerably higher concentrations of hydrogen sulfide.

When some of the hydrogen sulfide being formed is removed, as, for example, by zinc acetate in the upper arm of a Thunberg tube, or when it is substantially all removed by the presence of ferrous sulfate or cadmium carbonate in the nutrient medium, considerably larger amounts of total sulfide are formed than in corresponding controls. Large amounts of hydrogen sulfide in the culture medium thus seem to be a deterrent to further development.

Most rapid formation of hydrogen sulfide occurs when the inoculum is from a culture about two days old. As the age of inoculum increases from 2 to 20 days, the rate of formation of hydrogen sulfide in the new culture decreases so that 140 hours instead of 50 hours are required to reach the 50 per cent point in hydrogen sulfide concentration. When over 50 days old, a certain percentage of cultures lose viability entirely but growth may

take place in transfers from cultures over 300 days old. Survival rates are considerably higher when the cultures are held at 0.5° rather than at 30° C. There is no evidence that survival is mainly determined by the hydrogen sulfide present in the cultures.

LITERATURE CITED

1. BAARS, J. K. Over Sulfaatreductie door Bacteriën. 164 pp. Diss. Delft. 1930.
2. BUTLIN, K. R., MARY E. ADAMS, and MARGARET THOMAS. The isolation and cultivation of sulphate-reducing bacteria. Jour. Gen. Microbiol. **3**: 46–59. 1949.
3. MILLER, LAWRENCE P. Rapid formation of high concentrations of hydrogen sulfide by sulfate-reducing bacteria. Contrib. Boyce Thompson Inst. **15**: 437–465. 1949.

FORMATION OF METAL SULFIDES THROUGH THE ACTIVITIES OF SULFATE-REDUCING BACTERIA

LAWRENCE P. MILLER

Sulfate-reducing bacteria, *Desulfovibrio desulfuricans* (Beij.) K. and v. N., are widespread in their occurrence, being found in both salt and fresh water and in all soils generally. It has been suggested that they may have played an important role in the formation of the large commercial sulfur deposits and also of the metal sulfides (4). Black muds associated with the development of these bacteria owe their color to the precipitation of small amounts of iron present as ferrous sulfide. In tests reported in a previous paper (3) it was found that large amounts of ferrous sulfate were tolerated in culture media, and that under the conditions employed larger amounts of sulfate were reduced when ferrous sulfate was present in the media than in its absence. Similarly, cadmium carbonate could be added to the media whereupon the sulfide formed was converted into cadmium sulfide. Again more sulfide was formed in a given culture with cadmium carbonate present than when the sulfide remained in solution. These results suggested that compounds of other metals should be tested in this manner and it has been found that the sulfides of lead, zinc, antimony, bismuth, cobalt, and nickel could be obtained in culture media in which sulfate was being reduced. These findings therefore suggest that large-scale production through bacterial action of sulfides of some of the metals is possible as far as the necessary tolerance of the bacteria to relatively insoluble compounds of these metals is concerned. Whether there is any likelihood that such a process may have been associated with the formation of deposits of metal sulfides is a question beyond the scope of this paper but may warrant consideration by those interested in the genesis of ore deposits.

MATERIALS AND METHODS

Source of bacteria. Strains of bacteria used were those previously described (1). Insofar as these experiments are concerned no differences between strains were established and therefore specific strains are not referred to.

Nutrient media. Two basic nutrient media were used. One described previously as No. 220 contained the following ingredients in grams per liter: NH_4Cl, 1.00; $MgSO_4 \cdot 7H_2O$, 2.00; Na_2SO_4, 18.25; K_2HPO_4, 0.50; $CaCl_2 \cdot 2H_2O$, 0.10; sodium lactate, 21.0; NaCl, 10.0; $CaCO_3$, 1.0; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, trace. The other medium was similar in composition to No. 220 but contained more Na_2SO_4 and sodium lactate, namely totals of 27.38 and 32.0 g. respectively, and also 5 grams of yeast extract

Copyright, 1950, by Boyce Thompson Institute for Plant Research, Inc.

per liter, since yeast had been found to be a stimulant for the growth of the sulfate reducers (2). These media are referred to as A and B in the table given below. If all of the sodium lactate were used for the reduction of sulfate and the oxidation of the lactate proceeded only to acetate, quantities of sulfide equivalent to 3192 and 4864 mg. per liter of hydrogen sulfide theoretically can be formed in these two media, respectively.

Metal compounds. The following metal compounds were included in the tests: antimony trioxide, Sb_2O_3; bismuth subcarbonate, $(BiO)_2CO_3 \cdot H_2O$; cobalt carbonate, approximately $2CoCO_3 \cdot 3Co(OH)_2$; copper carbonate, $CuCO_3 \cdot Cu(OH)_2$; basic lead carbonate, approximately $2PbCO_3 \cdot Pb(OH)_2$; lead sulfate, $PbSO_4$; nickelous carbonate, technical; nickelous hydroxide, $Ni(OH)_2$; and zinc carbonate, approximately $2ZnCO_3 \cdot 3Zn(OH)_2$. These were obtained from the usual commercial sources except the lead sulfate and nickelous hydroxide which were prepared in the laboratory.

Culture techniques. The bacteria were cultured as previously described except that the medium was not freshly heated and cooled each day (1). The cultures were maintained either in glass stoppered 55-ml. Pyrex bottles or in 2-oz. flat prescription bottles varying in volume from 65 to 70 ml. and supplied with plastic caps. The quantity of metal compound used in each test was two grams per culture vessel. The amount of sulfide that could be precipitated by this quantity of additive depended, of course, upon the particular compound but, in general, exceeded the theoretical quantity that could be produced in the cultures except for the compounds of lead.

Determination of the sulfide formed. The sulfide produced was determined by conversion to hydrogen sulfide and reaction with an excess of iodine and back titration with sodium thiosulfate. Details of the procedure varied depending upon the properties of the metal sulfide in question. With some of the sulfides, titrations were carried out in 500-ml. glass-stoppered bottles. The contents of the culture vessels were rinsed into the titration bottles, acetic or hydrochloric acid added and an excess of iodine maintained in the bottles until all of the metal sulfide seemed to have been dissolved, after which the excess iodine was back titrated with sodium thiosulfate using starch as an indicator. This procedure could be used with the sulfides of cadmium (3), lead, zinc, and antimony. With antimony, higher concentrations of hydrochloric acid and a longer time were required. The ease with which the sulfides dissolved depended to some extent on the age of the cultures. No doubt the precipitates were most finely divided when first formed. This method did not give true results with a medium containing large amounts of ferrous sulfate and a large excess of calcium carbonate as described in a previous publication (3). However, under the conditions of the present experiments the correctness of the values, in

instances where there was any doubt, was checked by using the procedure described below.

An alternative procedure was to wash the contents of the culture vessel into a 500-ml. Erlenmeyer flask, acidify and pass a stream of nitrogen through the mixture in the flask and out through Van Slyke-Cullen tubes (5) containing 2 per cent zinc acetate. The zinc sulfide formed was then dissolved in hydrochloric acid and the hydrogen sulfide which was liberated titrated in the usual way. The nitrogen was passed through the culture until no more zinc sulfide was formed. With sulfides insoluble in acetic acid, it was possible to determine uncombined sulfide, by first determining the amount given off when acetic acid only was added. This alternative procedure was generally used with the sulfides of antimony, bismuth, cobalt, and nickel and with some of the other metals for purposes of comparison with the direct titration method. The sulfides of bismuth and cobalt are so difficultly soluble that under the conditions employed only a part of the sulfide present was recovered. Appropriate notations are made in the table given.

RESULTS

Growth was obtained in the presence of all the metals tested except copper. Only one compound of copper, $CuCO_3 \cdot Cu(OH)_2$, was included in the series. It would therefore be premature to conclude that the bacteria are necessarily more sensitive to copper than to the other metals tested. Development occurred readily in the presence of compounds of cadmium (3), iron (3), antimony, bismuth, and lead, somewhat less so with cobalt and zinc; and least readily in the presence of compounds of nickel. Some of the results are summarized in Table I. In general the quantity of sulfide produced was greater under these conditions when insoluble sulfides were precipitated than in the controls in which hydrogen sulfide accumulated. Extensive rate studies were not carried out but there was no evidence that the removal of sulfide increased the rate of sulfide production.

Although the metal compounds added were relatively insoluble in the media used hydrogen sulfide was removed fairly efficiently. In some instances data were obtained as to the quantity of free hydrogen sulfide in the cultures and these are shown in the table in footnotes *c*, *d*, *e*, *f*, and *g*.

DISCUSSION

The metal compounds added to the cultures are all relatively insoluble. It would not be expected that the bacteria would develop in appreciable concentrations of any of the metals included in these tests. Since the sulfides are still more insoluble, the compounds present were converted into the sulfides as hydrogen sulfide accumulated. Since the compounds were usually oxides, hydroxides, or carbonates, the other products of the reac-

tion with hydrogen sulfide were not harmful to the development of the bacteria.

In spite of the relative insolubility of the metal compounds added, some metal ions were in solution. This could be noted in some instances by the color imparted to the medium. The quantity of metal in solution when growth of the bacteria was initiated was variable since hydrogen sulfide in the inoculum precipitates the metals. The maximum concentra-

TABLE I

FORMATION OF METAL SULFIDES THROUGH THE ACTIVITIES OF SULFATE-REDUCING BACTERIA

Metal compound in medium	Total sulfide formed as mg./l. of H_2S			Metal compound in medium	Total sulfide formed as mg./l. of H_2S		
	Medium[a]	Metal compound present	Control		Medium[a]	Metal compound present	Control
Sb_2O_3	B	2437[b,c]	1862	$2PbCO_3 \cdot Pb(OH)_2$	A	2320	1908
	B	2927[d]	2208		A	2588	2086
	B	2795[e]	1751		A	1862	1915
	A	2149[b]	1998		A	1065	2045
	A	2456	1862		B	2617	2208
					B	3346	2085
$(BiO)_2CO_3 \cdot H_2O$	A	2707[b,f]	1908	$PbSO_4$	B	1756	2208
	B	3309[b,g]	2208		B	2449	1751
$CdCO_3$[h]	A	2666	1998	$Ni(OH)_2$	A	2134[b]	2401
	A	2808	2401				
	B	3339	2208				
$2CoCO_3 \cdot 3CO(OH)_2$	B	1506[b]	—	$2ZnCO_3 \cdot 3Zn(OH)_2$	B	3586	—
	B	2153[b]	2208		A	1425	1690

[a] For composition of media A and B see Materials and Methods section.
[b] Values low—metal sulfide did not dissolve completely.
[c] Of this quantity 718 mg./l. were present as free H_2S.
[d] Free H_2S, 280 mg./l.
[e] Free H_2S, 666 mg./l.
[f] Free H_2S, 766 mg./l.
[g] Free H_2S, 235 mg./l.
[h] Results with $CdCO_3$ published in a previous paper (3); a few values are included here for comparative purposes.

tion of the various metals tolerated by the bacteria has not been studied. Nevertheless, the results suggest at least a moderate tolerance to the metals tested.

Since hydrogen sulfide is known to be toxic to most forms of life, workers with sulfate-reducing bacteria have been tempted to assume that the hydrogen sulfide produced by these bacteria is deleterious to their further development. Results published in previous papers have indicated that the bacteria are not very sensitive to hydrogen sulfide and that rapid development of the bacteria is not prejudicial to the formation of high concentra-

tions (1, 3). However, when the sulfide produced is precipitated in the form of an insoluble metal sulfide, the total amount of sulfide formed is higher than in the controls. No evidence has so far been obtained that removal of the hydrogen sulfide increases the rate of formation and therefore rate of growth.

SUMMARY

Sulfides of antimony, bismuth, cobalt, cadmium, iron, lead, nickel, and zinc were formed as the result of the activities of sulfate-reducing bacteria, *Desulfovibrio desulfuricans* (Beij.) K. and v. N., when certain relatively insoluble compounds of these metals were present in the nutrient medium.

Growth took place most readily in the presence of the cadmium, iron, antimony, bismuth, and lead compounds tested, less readily with those of cobalt and zinc, and least readily in the presence of compounds of nickel. Of the metals studied results were completely negative only with $CuCO_3 \cdot Cu(OH)_2$, the only compound of copper tested.

In general, in these experiments in which the hydrogen sulfide produced was precipitated as an insoluble sulfide, the total amount of sulfide formed was larger than in the controls, thus indicating that the presence of high concentrations of hydrogen sulfide in the medium tends to retard further growth of the bacteria.

These results suggest that insofar as the tolerance of the sulfate reducers to certain metal compounds is concerned the suggestion that deposits of metal sulfides may have been the consequence of bacterial origin is feasible. Whether such an explanation of the formation of deposits of sulfide ores is tenable on the basis of other evidence available is beyond the scope of this paper but may warrant consideration by those especially interested in the genesis of ore deposits.

LITERATURE CITED

1. MILLER, LAWRENCE P. Rapid formation of high concentrations of hydrogen sulfide by sulfate-reducing bacteria. Contrib. Boyce Thompson Inst. **15**: 437–465. 1949.
2. ———. Stimulation of hydrogen sulfide production by sulfate-reducing bacteria. Contrib. Boyce Thompson Inst. **15**: 467–474. 1949.
3. ———. Tolerance of sulfate-reducing bacteria to hydrogen sulfide. Contrib. Boyce Thompson Inst. **16**: 73–83. 1950.
4. STARKEY, ROBERT L., and KENT M. WIGHT. Anerobic corrosion of iron in soil. 108 pp. American Gas Assoc., New York, N. Y. 1945.
5. VAN SLYKE, DONALD D., and GLENN E. CULLEN. A permanent preparation of urease and its use in the determination of urea. Jour. Biol. Chem. **19**: 211–228. 1914.

GROWTH AND REPRODUCTION OF WATER HYACINTH AND ALLIGATOR WEED AND THEIR CONTROL BY MEANS OF 2,4-D[1]

A. E. Hitchcock, P. W. Zimmerman, Henry Kirkpatrick, Jr., and T. T. Earle[2]

Experimental work dealing with the growth, reproduction, and practical control of water hyacinth (*Eichhornia crassipes* Solms.) and alligator weed (*Alternanthera philoxeroides* [Mart.] Griseb.) was started in April, 1948. Results of the first year's work were reported in 1949 (1), and a summary of later work, together with practical recommendations for controlling hyacinths in the waterways of Louisiana, appeared in the early part of 1950 (5). The present report describes the results of observations and experiments not previously reported and summarizes the work to date.

Results of experiments in 1949 confirmed those obtained in 1948 in showing that hyacinths in navigable waterways as well as in the experimental pits could be controlled by means of 2,4-D (2,4-dichlorophenoxyacetic acid). Due to the presence of overhanging trees and other hazards along the irregular margins of waterways, it was generally not possible to contact all hyacinths with a lethal dose of 2,4-D in a single application. Consequently, practical control of hyacinths consisted of applying with helicopter, truck-mounted, or boat-mounted equipment an initial spray containing a sufficient quantity of 2,4-D to cause sinking of 90 per cent or more of the plants, and a second follow-up spray to eliminate the hyacinths which had escaped being killed by the first treatment. The approximate minimum quantity of 2,4-D which proved effective under all conditions was 8 lb. per acre. The 4 lb. per acre dose was not consistently effective and the 2 lb. per acre dose was ineffective.

The results obtained in Florida by Seale and Allison (4) show that a dose of 1 lb. of 2,4-D per acre, applied in the form of the butyl ester in diesel oil from an airplane, removed on the average only 62 per cent of the hyacinth cover from infested canals. The authors speak of rapid regrowth occurring, especially during the winter season, which involves production of seedling hyacinths on the decaying mat of dead plants after spraying. Under the conditions existing in Louisiana during 1948 and 1949, a 1 to 2 lb. per acre dose of 2,4-D proved ineffective since only 10 to 50

[1] The investigations reported herewith were carried out by members of the staffs of Boyce Thompson Institute for Plant Research, Inc., Yonkers, N. Y.; Tulane University, New Orleans, La.; and the U. S. Corps of Engineers, New Orleans District. The cooperative project was made possible by a contract between Tulane University and the U. S. Corps of Engineers.

[2] Department of Botany, Tulane University, New Orleans, La.

Copyright, 1950, by Boyce Thompson Institute for Plant Research, Inc.

per cent of the hyacinths were killed. In fact, any treatment causing less than a 90 per cent reduction in hyacinth cover in our tests was considered ineffective on the basis of requiring a major instead of a minor follow-up patrol maintenance operation. In contrast to conditions in Florida, growth and vegetative propagation of hyacinths occurred at a very slow rate in Louisiana during the winter season (October through March) and seedling hyacinths were not found in any area which had received an 8 lb. per acre dose of 2,4-D.

The type of vegetation or other hazards along the margins of waterways infested with hyacinths will no doubt determine to a considerable extent what methods of treatment are most suitable for controlling hyacinths. There appear to be relatively few places in Louisiana where an airplane could operate as effectively as a helicopter. Another special condition in Louisiana is that alligator weed grows to a considerable extent in most of the waterways infested with hyacinths.

During 1949 an 8 lb. per acre dose of 2,4-D amine salt cleared the navigable waterways of both species. It was shown in 1948 that doses of 2 to 4 lb. of 2,4-D per acre applied in the summer caused relatively little damage to alligator weed growing in the experimental pits, but an 8 lb. per acre dose of 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) applied in August caused sinking of all plants (1, p. 387). The ester was more effective than the amine salt. With methods of treatment described in the present report, it is considered poor economy to attempt a saving in the cost of spraying operations by reducing the dose of 2,4-D substantially below 8 lb. per acre.

MATERIALS AND METHODS

Chemicals. In addition to the compounds and formulations used in 1948 (1), several new chemicals and prepared formulations were used in 1949. The following growth-regulating substances were obtained from the Dow Chemical Co., Midland, Mich.: sodium 4-chloro-*o*-toloxyacetate, sodium 2-methyl-4-chlorophenoxyacetate, sodium 1-naphthaleneacetate, sodium 4-chlorophenoxyacetate, and H-978, an ester of 2,4-D of low volatility. The following dyes were obtained from general Dyestuff Corp., New York City: Tapestry Red RN, Uranine WSS, Naphthol Yellow SXX New, Tapestry Printing Bright Yellow GG, and Metanil Yellow Extra Conc. Additional materials were sodium trichloroacetate (duPont), Triton B-1956 (Rohm and Haas), diesel oil, Shell gasoline dyes, and Ultra Wet. Since the alkanolamine and triethanolamine salts of 2,4-D were of equal activity (5, p. 78), no distinction is made between them in the present report, both being referred to as the amine salt. The alkanolamine salt of 2,4,5-T and the isopropyl esters of 2,4-D and 2,4,5-T were also used in the present tests. Nekal NS (General Dyestuff Corp.) was used as

a spreader at a concentration of 0.1 per cent in all spray solutions applied by ground equipment.

Spray equipment. Sprays were applied to hyacinths and alligator weed in experimental pits at a pressure of 25 to 30 lb. p.s.i. (per square inch) by the same equipment used in 1948 (1), with the exception that a five-nozzle instead of a four-nozzle boom was used in 1949. The pits were 15×30 ft. at the top, 10×10 ft. at the bottom, and 10 ft. at the deepest point. Spraying Systems' $1\frac{1}{4}$ inch TOC (Teejet off-center) boomjets were also used in applying sprays to plants in experimental pits and in canals. Four sizes of the TOC jets were used, and special equipment was designed to meet the output requirements of the different nozzle tips. A Porto Pump (Porto Pump Inc., Detroit, Mich.) having an output of 45 g.p.m. (gallons per minute) was fitted with a one inch by-pass valve and a one-half inch regulating valve to control the output at a given constant pressure. The requirements of the TOC jets varied from an output of 12 g.p.m. for the largest (No. 150) to 1.5 g.p.m. for the smallest (No. 20). A drum having a capacity of 50 gal. attached to the pump served as the spray tank. A Bean gun was also used with this equipment. For field work this equipment was mounted on a small truck or on a 20-ft. skiff powered by an outboard motor.

A Bean Junior Duplex pump with an output of 4 g.p.m. and a Bean Royal 35 pump with an output of 35 g.p.m. were used extensively in the large-scale field experiments. A Bean gun fitted with a No. 7 nozzle was used with the smaller pump and one fitted with a No. 16 nozzle was used with the larger pump. The Bean Royal 35 pump was attached to a tank having a capacity of 600 gal. which was mounted either on a large truck or on a small barge. Water for the sprays was pumped from the waterway by means of a suction pump having a capacity of 40 g.p.m.

A Bell helicopter, Model 47-D, equipped with the necessary spraying equipment and fitted with pontoons, was used extensively during 1949. This machine could land for reloading in a very limited area on land or water, or on a small barge. This type of helicopter is capable of spraying 600 acres or more per day, as compared to about 15 acres with boat-mounted equipment, and it can carry a spray load of 300 lb. The helicopter was fitted with two spray booms, each mounted at a 30° angle on either side of the fuselage. Each boom contained 41 tap bosses which were spaced three inches apart, alternating in a vertical and horizontal position, with the vertical ones pointed downward and the horizontal ones pointed toward the rear of the ship. The over-all length of the two booms from tip to tip was 23 ft. Additional spray equipment on the helicopter consisted of a centrifugal pump having an output of 30 g.p.m. at a pressure of 40 lb. p.s.i. Spraying Systems' Whirljet $\frac{1}{4}$ BAL No. 1 nozzles were used in all tests. The helicopter was flown at a speed of 30 m.p.h. (miles per hour)

when spraying and at altitudes of 10 to 80 ft. For some of the tests in areas inaccessible to land equipment a small barge was used on which the helicopter landed for reloading.

Coloration of spray solution. Various dyes were used in attempts to color the 2,4-D sprays. The five water-soluble dyes (General Dyestuff Corp.) colored a 1 per cent solution of 2,4-D, but the residue was not visible on the sprayed foliage of hyacinth or alligator weed. These dyes were not soluble in 20 to 40 per cent solutions of 2,4-D. The Orange and Red Shell gasoline dyes were soluble in 40 per cent 2,4-D, but the dyed sprays were not visible on hyacinth or alligator weed foliage. In cases where an injection system of adding concentrated 2,4-D directly to the water spray is used, the gasoline dyes would be of value in coloring the concentrate so that the operator would know whether the system was operating correctly by the presence or absence of color in the spray.

TABLE I

RELATIVE EFFECTIVENESS OF JUNE AND AUGUST SPRAYS OF THE AMINE SALT OF 2,4-D 60 TO 70 DAYS AFTER APPLICATION TO HYACINTHS GROWING IN PITS ONE TO TWO MONTHS BEFORE TREATMENT

Concn., %	Lb. per acre*	Percentage of plants sunk				No. of plants growing			
		June		August		June		August	
		1948 Ends	1949 Entire	1948 Entire	1949 Entire	1948 Ends	1949 Entire	1948 Entire	1949 Entire
0.3	2	50	99	98	98	2	6	1	3
0.6	4	50	99	98	100	0	8	0	0
1.2	8	98	100	100	100	0	0	0	0

* Applied at the rate of 78 gal. per acre.

RESPONSE OF PLANTS IN EXPERIMENTAL PITS

RESULTS WITH HYACINTHS

Effective doses of 2,4-D. Results of tests carried out in 1949 were in agreement with those obtained in 1948 in showing that 2,4-D was equally effective for killing and sinking hyacinths when all plants in the pit were treated (450 sq. ft.) as compared with treating 100 sq. ft. at the end portion (Table I). The relation between killing the aerial parts of hyacinths and the rate of sinking of the plants is shown in Figure 1 A. The curves represent the average results for all treatments applied to entire pits in 1949. These data (Fig. 1 A) show that 95 to 100 per cent top killing occurred in 15 to 35 days as compared with the equivalent percentage of sinking in 65 to 100 days.

The rate at which hyacinths sank was influenced to a much greater extent by the dose of 2,4-D than was the rate of top killing (Fig. 1 A).

Since the rate of delivery was held constant at 78 gal. per acre, the dose ratio was the same for concentration (0.3, 0.6, and 1.2 per cent) and rate of application (2, 4, and 8 lb. per acre). There was no noticeable increase in

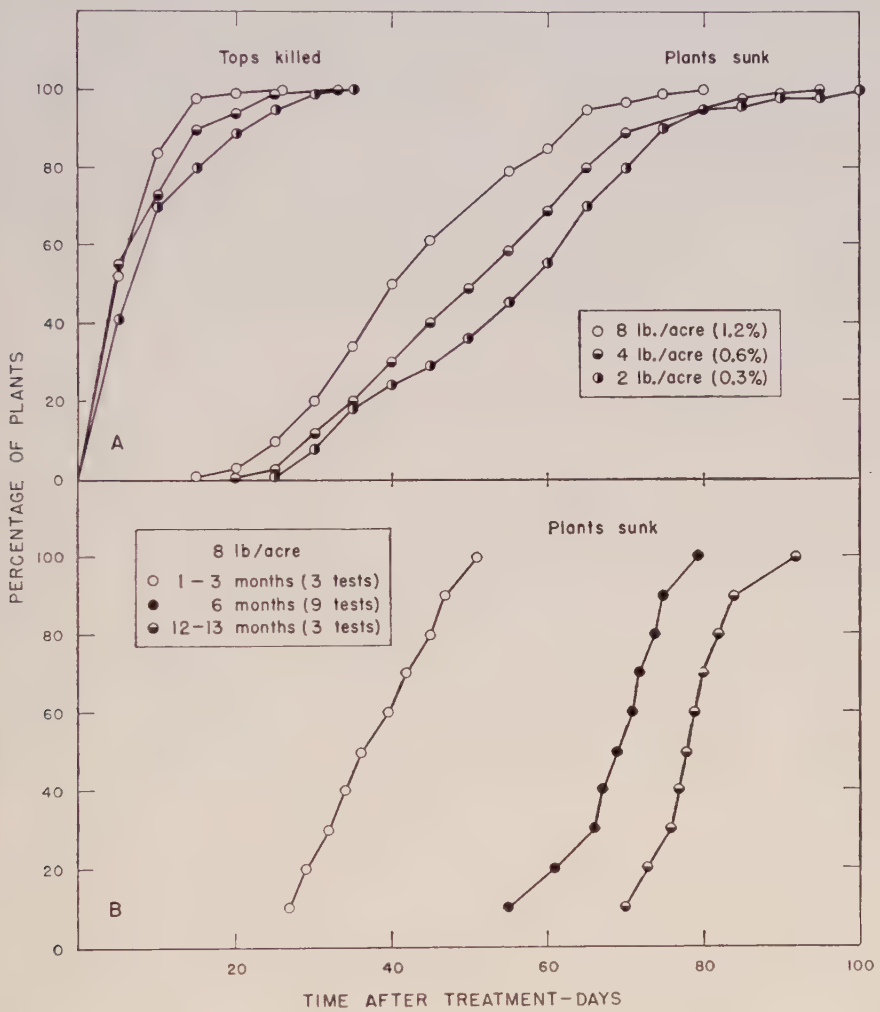


FIGURE 1. A. Average rate of killing and sinking of hyacinths in experimental pits after treatment with different doses of the 2,4-D amine salt. B. Average rate of sinking of hyacinths according to the time the plants were growing in the experimental pits before treatment.

rate of top killing with increased rates of application from 2 to 8 lb. per acre of 2,4-D. However, an increase in rate of sinking was induced by an increase in rates of application. It is evident from these results (Fig. 1 A)

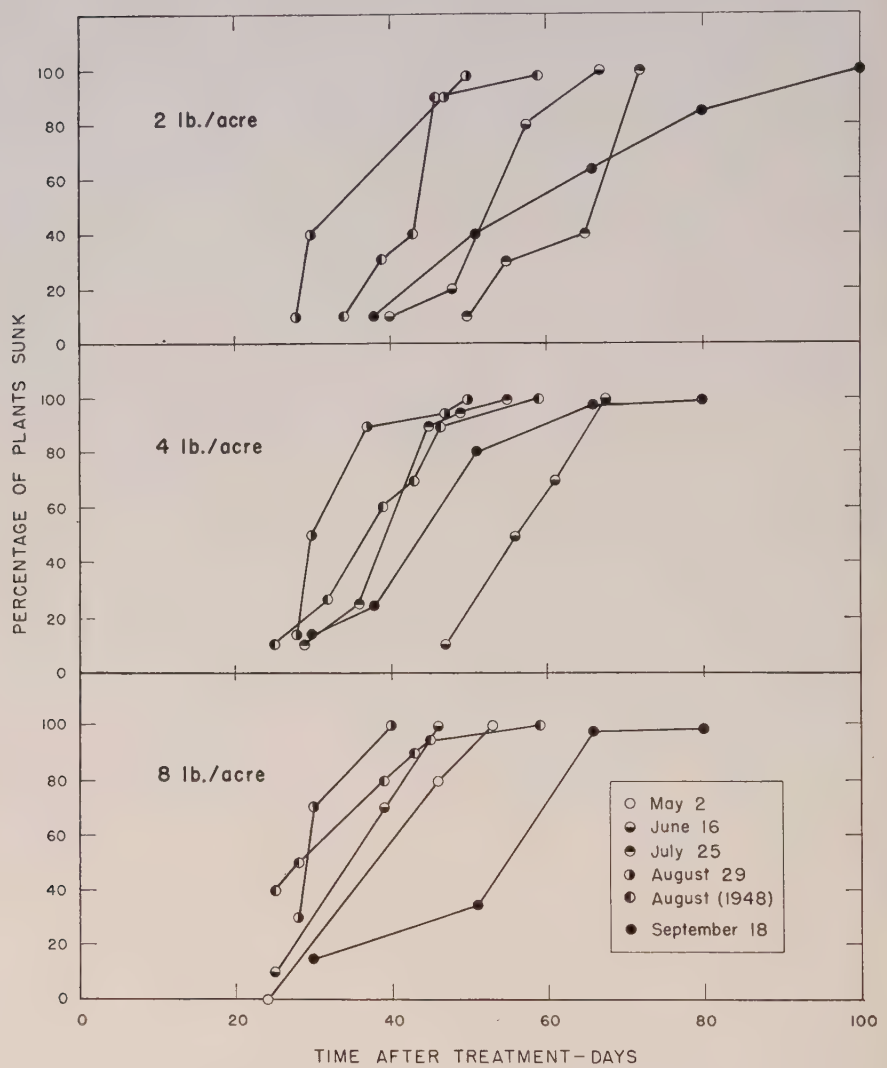


FIGURE 2. Rate of sinking of hyacinths in experimental pits after treatment with different doses of the 2,4-D amine salt at different times of the year.

that the percentage of hyacinths sunk is a more reliable criterion than the percentage of plants with tops killed for determining differences in lethal effects caused by 2,4-D. The 8 lb. dose of 2,4-D killed all hyacinths on the average of 15 to 20 days sooner than the 2 to 4 lb. doses. Other examples of the greater effectiveness of the 8 lb. per acre dose as compared with the 2 to 4 lb. doses are shown in Table I and will be referred to in other sections.

Seasonal effects. Since the time for all plants to sink ranged from 40 to

100 days (Fig. 2), it appeared that factors other than the dose of 2,4-D were affecting the rate of sinking. Data in Figure 2 show that hyacinths treated in August sank 30 to 50 days sooner than those receiving a similar treatment in September. This applied to hyacinths which had been growing in the pits for one to two months prior to treatment. Similar plants treated in May, June, or July gave variable results according to the dose of 2,4-D, but in each case the time for all plants to sink was intermediate between that for the August and September treatments. Whereas in August the 2 to 8 lb. per acre rates of application were about equally effective, in June the 8 lb. dose was considerably more effective than the 2 to 4 lb.

TABLE II

EFFECT OF WASHING FOLIAGE OF HYACINTHS AFTER TREATMENT WITH 8 LB. OF 2,4-D APPLIED AT THE RATE OF 78 GAL. PER ACRE TO PLANTS GROWING IN THE PITS 12 MONTHS BEFORE BEING TREATED

Interval before washing,* minutes	Spreader		Percentage of plants sunk after days indicated			
	Name	Concn., %	70	80	90	112
0	Nekal NS	0.25	0	80	99	100
	Triton 1956-B	0.05	0	50	85	100
15	Nekal NS	0.25	5	10	20	60
	Triton 1956-B	0.05	5	20	35	90
30	Nekal NS	0.25	10	10	10	90
	Triton 1956-B	0.05	10	30	60	98

* Washed by means of a fire hose.

doses. Similar results (1, p. 371, 372) were obtained in 1948 (Fig. 2). Thus a 2 lb. per acre dose applied in August was as effective for sinking all hyacinths as an 8 lb. dose applied in June.

Age of plants in pits. The length of time the hyacinths had grown in the pits before being treated was also an important limiting factor (Figs. 1 B and 3). For example, hyacinths growing in the pits 1 to 3 months before treatment with an 8 lb. dose of 2,4-D sank 28 days sooner than similarly treated plants that were in the pits 6 months, and 42 days sooner than plants growing in the pits for 12 to 13 months (Fig. 1 B). Similar results for plants 12 months in the pits as compared with 1 to 2 months are shown in Tables I, II, and III respectively. In this case the hyacinths in the pits for 12 months required an average of 40 days longer to sink as compared with the plants which were in the pits for only 1 to 2 months before treatment.

Relative effectiveness of 2,4-D and 2,4,5-T. Results in 1949 (Table III) confirmed those obtained in 1948 (1, p. 377) in showing that the isopropyl

esters and amine salts of 2,4-D and 2,4,5-T were of about equal activity for killing and sinking hyacinths, provided the spray solution contained a minimum of about 1.0 per cent 2,4-D acid equivalent and was delivered at the rate of 8 lb. per acre. This equivalency did not hold for rates of 2 to 4 lb. per acre in which case the amine salts were more effective than

TABLE III

COMPARATIVE EFFECTIVENESS OF AMINE SALTS AND ISOPROPYL ESTERS OF 2,4-D AND 2,4,5-T 50 DAYS AFTER APPLICATION TO HYACINTHS GROWING IN PITS ONE MONTH BEFORE TREATMENT

Concn., %	Lb. per acre*	Percentage of plants sunk				No. of plants growing			
		2,4-D		2,4,5-T		2,4-D		2,4,5-T	
		Salt	Ester	Salt	Ester	Salt	Ester	Salt	Ester
0.3	2	98	90	95	0	3	25	50	7000
0.6	4	100	99	100	60	0	5	0	200
1.2	8	100	100	100	100	0	0	0	0

* Sprays applied August 29, 1949, to entire pits at the rate of 78 gal. per acre.

TABLE IV

COMPARATIVE EFFECTIVENESS OF 2,4-D SALT FORMULATIONS APPLIED BY MEANS OF A TOC JET NO. 80 NOZZLE ON APRIL 7, 1949, TO HYACINTHS GROWING IN PITS SEVEN MONTHS BEFORE TREATMENT

2,4-D 1%*	Spreader		No. of plants growing after days indicated		
	Name	Concn., %	39	63	67
Sodium salt	None	0	40	70	90
	Nekal NS	0.25	3	0	0
	Ultra Wet	0.05	4	10	8
Amine salt	None	0	5	0	0
	Nekal NS	0.25	0	0	0
	Ultra Wet	0.05	8	5	3

* Approximately 8 lb. per acre.

their corresponding esters, but the ester of 2,4-D was considerably more effective than the ester of 2,4,5-T (Table III).

Spreaders and adjuvants. Nekal NS was used as a wetting agent in all 2,4-D spray solutions except for the few cases in which Nekal was compared with other wetting agents (Table IV). The effectiveness of a 2,4-D amine salt formulation was not increased noticeably by the addition of a wetting agent when the spray solution was delivered with low-pressure equipment (25 to 30 lb. p.s.i.) and under conditions in which rain did not interfere with the tests. This applied also to sprays delivered at a pressure of 200 lb. p.s.i. with TOC jets (Table IV). The washing action of rain within

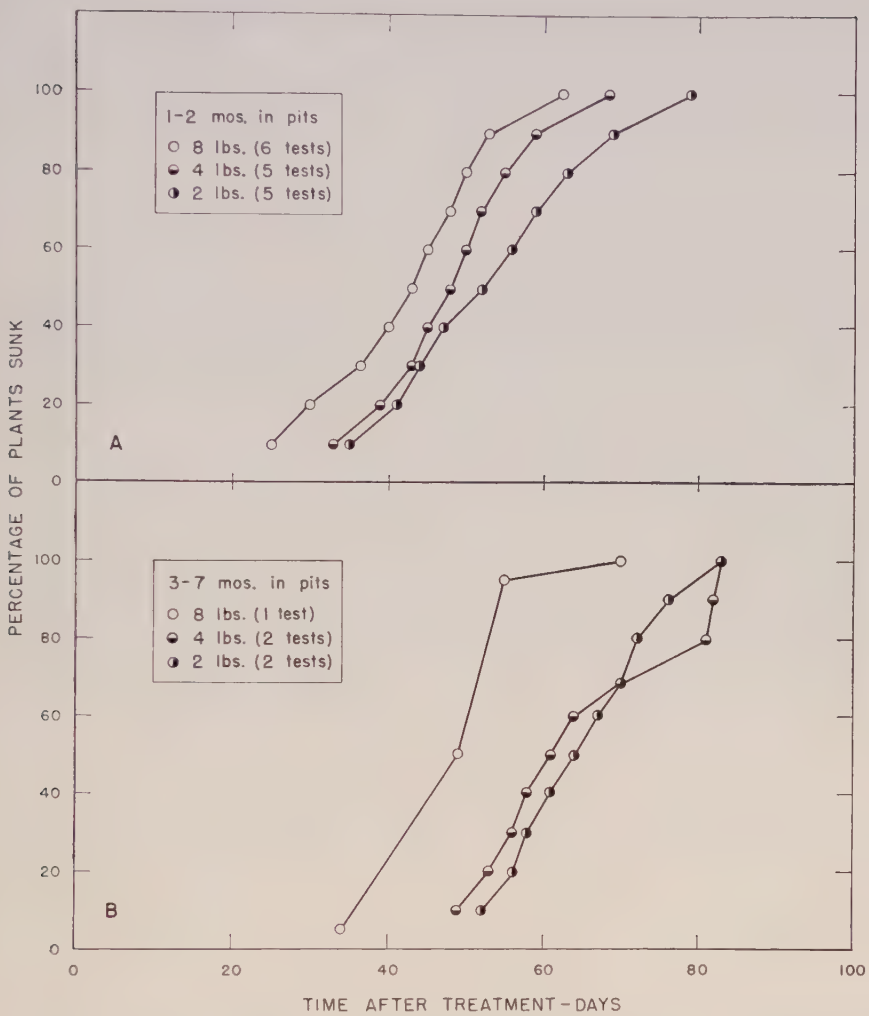


FIGURE 3. Rate of sinking of hyacinths in experimental pits after treatment with different doses of the 2,4-D amine salt and according to the time the plants were growing in the pits before treatment.

30 minutes after treatment generally reduced the effectiveness of a 2,4-D spray. Under these special conditions some wetting agents may be more effective than others, as indicated by the results obtained with Nekal NS and Triton 1956-B (Table II). Presumably the greater effectiveness of Triton 1956-B was due to its greater adhesive properties as compared with Nekal NS. Results in Table IV show that the sodium salt of 2,4-D was much more effective when a wetting agent was added. In this case Nekal NS proved more effective than Ultra Wet. The addition of 0.05 per cent

Ultra Wet to the amine salt of 2,4-D caused a slight reduction in the effectiveness of the spray treatment, perhaps due to an excessive run-off that might result in leaving less 2,4-D residue on the smooth surface of the hyacinth foliage. Although in these tests and in those carried out in 1948 (1, p. 367) the effectiveness of the 2,4-D amine salt formulation was not increased noticeably by the addition of a wetting agent, the use of a wetting agent might be justified in large-scale spraying with a gun-type sprayer

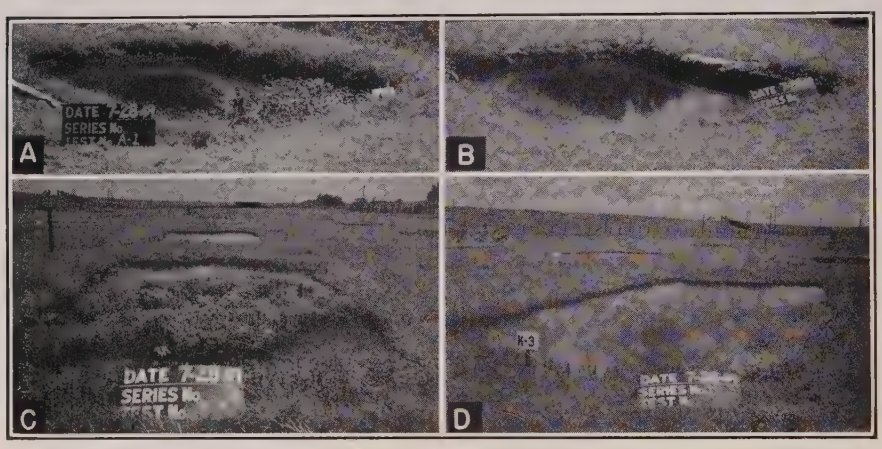


FIGURE 4. Regrowth from chopped hyacinths and alligator weed after treatment with an 8 lb. per acre dose of the amine salt of 2,4-D as compared with chopped material not treated. A. Regrowth in 28 days from chopped hyacinths not treated. B. Sinking of all chopped hyacinth material 23 days after treatment. C. Regrowth in 63 days from chopped alligator weed not treated. D. Sinking of chopped alligator weed 57 days after treatment. Note shoots growing from a few stem pieces which rooted in shallow (foreground) end of pit.

on the basis of aiding the operator to see which areas have been adequately covered.

In 1948 the addition of 1 to 10 per cent of sodium trichloroacetate to an isopropyl ester formulation of 2,4-D appeared to increase the effectiveness of the treatment (1, p. 378). Similar additive effects were not obtained in 1949 when the amine salt was used instead of the ester. The use of 1 to 10 per cent of diesel oil with the amine salt caused a reduction in the rate of sinking. These results indicate that water-soluble forms of 2,4-D may not be compatible with oil or oil-soluble adjuvants unless suitable emulsifying agents are present.

Other hormone herbicides. Several hormone-type herbicides not tested in 1948 were compared with 2,4-D in tests carried out in 1949. At a concentration of 1.2 per cent sodium 2-methyl-4-chlorophenoxyacetate and Dow's low volatile ester (H-978) were equivalent in activity to the amine

salt of 2,4-D. Sodium 1-naphthaleneacetate did not kill hyacinths and sodium 4-chlorophenoxyacetate killed only about 50 per cent of the plants. There was continuous flowering of hyacinths throughout the summer in pits treated with sodium 1-naphthaleneacetate.

Effect of 2,4-D on chopped hyacinths. For many years it has been a common practice in Louisiana to open channels in hyacinth jams by means of saw-boats. The number of times the saw-boat passes through a given area determines the size of the pieces. Much of the chopped material floats, including the bud-containing rhizomes which are capable of forming new plants. An example of regrowth from boat-chopped material is shown in Figure 4 A. Hand-chopped plants acted in a similar manner. Treatment of the hand-chopped and saw-boat-chopped hyacinth material

TABLE V

EFFECTIVENESS OF 2,4-D IN KILLING AND SINKING CHOPPED ALLIGATOR WEED WHEN THE MATERIAL WAS TREATED FIVE DAYS AFTER BEING PLACED IN THE EXPERIMENTAL PITS

Treatment	Average length of cut pieces, inches	Pit area covered (sq. ft.) after days indicated			
		0	50	100	150
Amine salt of 2,4-D 1.2% 8 lb./acre	.2	225	6	0	20
	5	400	200	45	45
None	2	225	9	180	225
	5	400	200	270	270

with 2,4-D within two weeks after placing the material in the experimental pits killed all portions of the plants, and consequently there was no regrowth in the treated pits (Fig. 4 B). In contrast, after three weeks there were 700 hyacinths growing from the non-treated material chopped by saw-boats and 200 hyacinths from the hand-chopped control material. At the end of three months the control hand-chopped material had produced 2,500 hyacinths. These results show that hyacinths are not likely to be eradicated or controlled by chopping the plants into small pieces, but eradication can be accomplished by spraying the chopped material with an effective dose of 2,4-D, as in the case of intact plants. The same dose of 2,4-D (8 lb. per acre) which eradicated chopped hyacinths was also effective for eradicating chopped alligator weed (Fig. 4 D and Table V).

Penfound and Earle reported that hyacinths segmented by hand or by saw-boats resprouted abundantly but that treatment of the chopped material with 1 lb. per acre of the sodium salt of 2,4-D did not affect the rate of regeneration (2, p. 463). Their conclusion that it would be useless to spray the segmented hyacinth material is of course based upon the use

of a dose of 2,4-D which according to the present results would be ineffective for killing intact hyacinths.

Potential reinfestation from surviving plants. The experimental pits held a maximum of about 8,000 hyacinths. On the basis of this estimate and of estimates made for hyacinths throughout the Spillway area, there would be about 800,000 hyacinths per acre in any area where the plants were packed tightly together. This value is four times greater than the estimate of hyacinths growing in canals in Florida (4). Since hyacinths in experimental pits were observed to increase by off-shoot formation at the rate of 1,000 times in 50 days (1, p. 383), it is evident that in practical control

TABLE VI

RELATIVE EFFECTIVENESS OF 1.0 PER CENT 2,4-D FOR CAUSING HYACINTHS TO SINK WHEN THE SPRAY SOLUTION IS DELIVERED FROM TOC JETS OF DIFFERENT SIZE ATTACHED TO A JUNIOR BEAN DUPLEX PUMP OPERATED AT A PRESSURE OF 150 LB. P.S.I.

TOC jet No.	Lb. per acre	Percentage of plants sunk after days indicated			No. of plants growing after days indicated		
		37	61	73	37	61	73
20	1	0	20	60	4800	6000	7200
40	2	0	80	90	125	200	800
80	4	0	80	90	80	25	4
150*	4	20	99	99	25	13	3

* 14 g.p.m. output of pump required for this nozzle, but 4 g.p.m. was used.

operations an effort should be made to use treatments which will kill every plant.

Even though all hyacinths in a pit were eradicated by 8 lb. per acre of 2,4-D in most of the tests, it is not likely that this can be done in large-scale practical control operations where it is difficult to contact all plants with the spray solution. Consequently, as will be shown in a later section, an initial treatment in the field may be considered effective if it causes sinking of 90 per cent or more of the hyacinths, since a properly-timed follow-up patrol maintenance spray caused sinking of most plants not eradicated by the first spray.

Evaluating different treatments on the basis of the percentage of plants sunk is not likely to show significant differences if all values lie in the range 90 to 100 per cent. In this case the number of plants growing some days after treatment served as a more suitable criterion since the values range from less than 10 to more than 1,000 (Table VI). For example, the 2 lb. and 4 lb. doses of 2,4-D each caused an estimated sinking of 90 per cent of the original number of hyacinths in a pit area of about 450 sq. ft. The actual number of plants including new offshoots 73 days after treatment was 800 in the pit receiving the 2 lb. per acre dose and 4 in the

pit receiving the 4 lb. per acre dose. When considering large-scale operations, the potential reinfestation from 800 plants in 50 days would be sufficient to cover an acre of water as compared with one-eleventh acre from 4 plants. Another striking example of how deceiving comparative results may be when expressed on the basis of small-scale tests as compared with large-scale tests is illustrated by an experiment in which the average results of six pit tests showed that the number of hyacinths not sunk was 16.8, 6.3, and 0.2 respectively in pits receiving 2, 4, and 8 lb. per acre doses of 2,4-D. The corresponding percentages for the above number of plants not sunk are 0.21, 0.08, and 0.01 based on the original number of 8,000 plants per pit. When the number of plants remaining in the pits is adjusted to an area of one acre, then the calculated area which the remaining hyacinths would occupy in two months is 3.15, 1.18, and 0.04 acres respectively for the 2, 4, and 8 lb. per acre doses of 2,4-D. These differences emphasize the advantage of using the 8 lb. per acre dose of 2,4-D in large-scale operations.

Relative toxicity of 2,4-D and other chemicals to animal life. There was no evidence of 2,4-D being toxic to animal life in the experimental pits or in any of the other treated areas. Although livestock had access to the canals which were sprayed with 2,4-D, there was no evidence of toxicity due to eating the foliage of treated hyacinths or from drinking the water. This is in contrast to killing of fish, snakes, turtles, frogs, tadpoles, and crawfish within 24 hours after spraying alligator weed in a pit with 3.2 per cent of Dow's General Weed Killer. All of the animals killed rose to the surface where they floated until removed. Ten per cent sodium pentachlorophenate killed fish within 10 minutes after spraying the solution on alligator weed in a pit. The fish at first were extremely agitated, jumping above the surface and then turning on their sides where they remained for a few minutes before sinking to the bottom of the pit.

RESULTS WITH ALLIGATOR WEED

Treatments with the amine salt of 2,4-D which were effective in 1948 for killing and sinking hyacinths within three months were not similarly effective for eradicating alligator weed growing in the experimental pits (1, p. 387). The isopropyl ester of 2,4,5-T applied as a 17.5 per cent solution at the rate of 8 lb. per acre on August 31, 1948, was the only treatment which resulted in the sinking of at least 95 per cent of the plants within three months. A similar treatment with the amine salt of 2,4,5-T killed all foliage and inhibited shoot growth for a period of about eight months without causing complete sinking of all the stems. After a period of 21 months practically all of the plants sank, as in the case of those treated with the ester of 2,4,5-T, but with the difference that the amine salt required 18 more months to accomplish the same result.

The fact that alligator weed growing in the experimental pits would sink eventually after a period of six months or longer as the result of a single 8 lb. per acre treatment, was not anticipated when the tests in 1949 were planned and carried out as indicated in Table VII. In the 1949 tests emphasis was placed on the application of at least two sprays with the idea

TABLE VII

SUMMARY OF TREATMENTS IN 1949 IN WHICH A 1.2 PER CENT SOLUTION OF THE HERBICIDE WAS APPLIED AT THE RATE OF 8 LB. PER ACRE TO ALLIGATOR WEED GROWING IN EXPERIMENTAL PITS

Time of first and last treatment		Remarks	Clear water May, 1950, %	Time of first and last treatment		Remarks	Clear water May, 1950, %
2,4-D amine salt				2,4-D isopropyl ester			
Mar. 22	Aug. 29	—	90	May 5	Aug. 30	—	40
May 5	Aug. 30	—	95	May 5	Aug. 30	10% Diesel oil	100
May 5	Aug. 30	5% Diesel oil	99	July 14	Sept. 18	Rain 90 min.	100
May 5	Aug. 30	10% Diesel oil	100	July 14	Sept. 18	Rain 10 min.	85
June 16	Aug. 17	—	25	Aug. 17	—	—	90
June 27	—	—	75	Aug. 17	—	H-978	95
July 14	Sept. 18	Rain 2 hr.	50	Sept. 18	—	—	95
July 25	Sept. 18	5% Diesel oil	60	Sept. 18	—	5% Diesel oil	100
Aug. 17	—	—	85	Sept. 18	—	H-978	99
Aug. 29	—	—	75	Sept. 18	—	5% Diesel oil	85*
Sept. 18	—	—	50	Oct. 14	—	—	100
Sept. 18	—	5% Diesel oil	75	Oct. 14	—	H-978	75
Oct. 14	—	—	100				
Average			65	Average			89
2,4,5-T amine salt				2,4,5-T isopropyl ester			
Mar. 22	Aug. 29	—	99	May 5	Aug. 30	—	100
Aug. 17	—	—	80	May 5	Aug. 30	10% Diesel oil	90
Oct. 14	—	—	99	July 14	Sept. 18	Rain 20 min.	100
				Sept. 18	—	—	95
				Sept. 18	—	5% Diesel oil	100
				Oct. 14	—	—	90
Average			93	Average			96

* H-978 used in this test.

that the second spray would constitute an effective patrol maintenance treatment for alligator weed as in the case of hyacinth (5, p. 77). Although this proved to be true in large-scale practical control operations, the same treatments were not equally effective on alligator weed growing in the experimental pits where lack of disturbance and the presence of shallow water at one end of the pit delayed sinking of a substantial proportion of the stems.

The response of alligator weed in the experimental pits to two or three

successive treatments at intervals of 55 to 66 days, as compared with a single treatment, shows that the percentage of plant material sunk varied with the kind and formulation of herbicide (Table VIII) and the time of

TABLE VIII

RELATIVE EFFECTIVENESS OF THE AMINE SALTS AND ISOPROPYL ESTERS OF 2,4-D AND 2,4,5-T FOR KILLING AND SINKING ALLIGATOR WEED WHEN ONE TO THREE SPRAYS WERE APPLIED TO THE PLANTS AT THE RATE OF 8 LB. ACID EQUIVALENT (1.2 PER CENT) PER ACRE

Time of treatment	Percentage of plants sunk after final treatment in time indicated							
	61–67 Days				7–9 Months			
	2,4-D		2,4,5-T		2,4-D		2,4,5-T	
	Amine salt	Ester	Amine salt	Ester	Amine salt	Ester	Amine salt	Ester
May, July, August 30	60	15	—	98	95	40	—	100
August 30	0	0	0	0	85	90	80	20*
July, September 18	40	99	—	99	50	100	—	100
September 18	40	20	—	50	50	95	—	95
October 14	60	25	25	20	100	100	99	90

* A second spray with the isopropyl ester of 2,4-D applied in September.

TABLE IX

RELATIVE EFFECTIVENESS ON ALLIGATOR WEED OF THE FIRST AND LAST OF SEVERAL SUCCESSIVE TREATMENTS, EACH CONSISTING OF 8 LB. OF THE 2,4-D AMINE SALT PER ACRE

No. of treatments	Date of first treatment	Date of last treatment	Percentage of plants sunk in 7 to 9 months	
			After first treatment	After last treatment
4	Mar. 22	August	25	90
3	May 5	August	60	95
2	June 16	August	0	25
2	July 14	September	10	50
2	July 25	September	15	60
1	Aug. 17	—	85	—
1	Aug. 29	—	75	—
1	Sept. 18	—	50	—
1	Oct. 14	—	100	—

year the plants were treated (Table IX). The number of treatments appeared to be of less importance (Tables VIII and IX). Single treatments with both formulations of 2,4-D and 2,4,5-T were effective when applied in September or October (Table VIII). The time of year was an important factor for all formulations except the ester of 2,4,5-T. The greater effec-

tiveness of the ester of 2,4,5-T for killing alligator weed in experimental pits confirms the previous results obtained in 1948 (1).

Adding 5 per cent of diesel oil to the amine salt and isopropyl ester formulations of 2,4-D resulted in a higher percentage sinking (Table X). Similar consistent results were not obtained when diesel oil was added to esters of 2,4,5-T and the low volatile ester, H-978 (Table VII). Since by proper timing of the sprays, eradication of alligator weed was accomplished both in the experimental pits and in large waterways without the use of diesel oil as an adjuvant, there would appear to be no special advantage in the use of this oil in practical control operations.

The fact that the amine salt of 2,4-D applied at the 8-lb. rate in October caused sinking of all alligator weed is of considerable practical impor-

TABLE X

EFFECT ON ALLIGATOR WEED OF ADDING DIESEL OIL TO 2,4-D AND 2,4,5-T APPLIED AT THE RATE OF 8 LB. (1.2 PER CENT) PER ACRE

Formulation		Percentage of plants sunk, May, 1950			
		One treatment, September, 1949		Three treatments, May, July, August, 1949	
		Diesel oil, %		Diesel oil, %*	
		0	5	0	10
Amine salt	2,4-D	50	75	95	100
Isopropyl ester	2,4-D	95	100	40	100
	2,4,5-T	95	100	100	90

* Diesel oil used only in May treatment.

tance since the same treatment was shown to be effective for sinking hyacinths at any time of the year. It is to be recalled, however, that in large-scale field operations treatment of a mixed growth of hyacinths and alligator weed with the amine salt of 2,4-D in April and in June caused sinking of all plants of both species by July 28 (5, p. 77). As mentioned previously, alligator weed in experimental pits was more difficult to kill than plants growing in deep waterways. The reason for this difference became apparent after the growth habits of alligator weed were better known.

Unlike water hyacinth, the alligator weed is not a floating species which can grow and survive without having its roots anchored in soil or in a similar substrate which supports root growth. Thus the growth of alligator weed from the banks of waterways out into the water is limited to the depth of water that permits roots to anchor in soil which generally means a depth of less than 5 ft. In the observed cases, the outer margin of a pure stand of alligator weed stopped growing and generally turned yellowish when the

plants reached water having a depth of 10 ft. or more. Plants at the outward margin of such a fringe growth broke away from the main mass in clumps ranging from several to many feet in diameter as a result of wind action or other forms of disturbance. These free floating clumps failed to grow unless they reached and remained in shallow water or against a soil bank long enough for the roots to become anchored in soil. These observed limitations were confirmed experimentally by isolating a group of alligator weed in water having a depth of 5 to 7 ft. under the plants during the test period of 21 months, at the end of which the plants had died as a result of not securing a root anchorage in soil. The appearance of these plants at the start and after 8 to 10 months shows that there was no substantial increase in the size of the group even when surrounded by hyacinths (Fig. 5).

When alligator weed was transferred to the experimental pits, the plants grew and became established at a slow rate since more than half of the pit area contained water which was 5 to 10 ft. in depth. Eventually some of the plants became anchored in soil along the three vertical banks and also in the shallow end of the pit where the fourth bank sloped into the water. The maximum growth attained by alligator weed in experimental pits was not so vigorous as in tree-shaded bayous and canals.

Alligator weed underwent the following successive changes after treatment with an effective dose of 2,4-D or 2,4,5-T: killing of all foliage and the upper parts of shoots; flattening of the aerial stems to a horizontal position; reduction in thickness of the mat of stems first above and then below the water level to a thin floating layer as shown in Figure 6 B; growth of spindly new shoots from submerged stems starting at about the time noticeable reduction in the area of the floating material became evident; and continued sinking of the floating material until all disappeared except in a few cases, as shown in Figure 4 D in the foreground end. A higher percentage of treated plants or stems survived by becoming anchored in soil at the shallow end of the pits than in large waterways, where there was a more pronounced disturbance resulting from wind and water currents.

The difference in the capacity of a few isolated plants of alligator weed and a few hyacinths to reinfest a given area is illustrated in Figure 5. The few hyacinths dropped accidentally along the banks of the pit in September (Fig. 5 A) reproduced slowly by means of offshoots until the following spring when rapid vegetative reproduction resulted in covering the entire pit area, most of which occurred in 2.5 months. In contrast there was no perceptible increase in the area covered by the alligator weed. These results show that alligator weed does not grow over or become intermingled with hyacinths unless the plants are anchored in soil or in substrate which supports root growth. The rapid rate of reinfestation by hyacinths in this case

FIGURE 5. Relative difference in spread of alligator weed and hyacinths in deep water. A. Alligator weed in wire basket held by cables in center of pit where water was 8 to 10 ft. deep throughout test period starting September, 1948. Three hyacinths are in grass at edge of water. B. Appearance of pit in May, 1949, showing alligator weed at a standstill and hyacinths in the process of rapid vegetative reproduction. C. Appearance of pit in July, 1949, showing alligator weed at a standstill surrounded by hyacinths which have occupied the remainder of the water surface.

FIGURE 6. Representative response of alligator weed in experimental pits after treatment with an 8 lb. per acre dose of the esters of 2,4-D or 2,4,5-T. A. Before treatment. B. Mat of floating and submerged leafless stems as they appear three to four weeks after treatment when the material starts to sink. C. Sinking of all except a small amount of material in the shallow (foreground) end of the pit which occurs within three months.

is similar to that described in a previous section (*Potential reinfestation from surviving plants*).

Prior to 1948 temporary channels were made through alligator weed by saw-boats which chopped the plants into small pieces. Most of the chopped material remained on the surface or was partially submerged. There has been considerable controversy over the question of whether the chopped alligator weed can grow and reestablish itself on a large enough scale to be regarded as an important source for reinfesting the original area or new areas where the floating material is carried by wind and water currents. Results of tests in which chopped alligator weed was thrown on the water in the experimental pits showed that the amount of material which sank and the regrowth from that which remained afloat varied according to the average size of the chopped pieces (Table V). After five months, regrowth from pieces two inches in length had reinfested an area equal to that originally occupied by the chopped material. In a similar time regrowth from pieces five inches long reinfested about two-thirds of the original area (Fig. 4 C). These results show that chopped alligator weed has the potential capacity to reinfest within a few months an area equal to more than 50 per cent of the original area covered by the floating material.

Treatment of chopped alligator weed with an 8 lb. per acre dose of 2,4-D resulted in eradicating most of the material within two months (Table V and Fig. 4 D). A similar treatment was equally effective on chopped hyacinths, but in this case the material sank in 23 days (Fig. 4 B).

Several different chemicals were applied during the summer to a mixed growth of alligator weed and hyacinths growing in the experimental pits. A given treatment with 2,4-D caused each species to sink in the same time as when grown separately in the experimental pits, namely, within three months for hyacinths and six months or longer for alligator weed. The amine salt was more effective than the H-978 ester in sinking alligator weed when the sprays were applied June 27, but the two chemicals were equally effective for sinking hyacinths. When the mixed growth of plants was treated in May with sodium 2-methyl-4-chlorophenoxyacetate, all hyacinths sank in 100 days as compared with 80 days for the amine salt of 2,4-D. A 1.2 per cent solution of sodium 1-naphthaleneacetate inhibited the growth of both species but caused neither killing of the foliage nor sinking of the plants. Hyacinths continued to flower in this pit at irregular intervals between the regular flowering cycles when flowering did not occur elsewhere in the Bonnet Carré Spillway area.

A few of the alligator weed plants treated with sodium 1-naphthaleneacetate set seed which were oval in shape, approximately 2 mm. in length, and equipped with two wings arranged longitudinally on opposite sides. Under natural conditions alligator weed does not normally set seed,

although the plant is a prolific flowering type. Seed set was not observed anywhere except on the treated plants just mentioned.

PRACTICAL CONTROL OF HYACINTHS AND ALLIGATOR WEED

RESULTS WITH GROUND EQUIPMENT

Comparative effectiveness of different sprayers. Results with spray equipment used in practical control tests varied according to the type of sprayer, speed of spraying, rate of delivering the spray solution, and the dose of 2,4-D. The spray equipment used prior to June, 1949, consisted of a gun-type nozzle equipped with a 7/64 inch tip that delivered 4 g.p.m. when operated by a Bean Junior Duplex pump. The effective range of this equipment was 15 to 25 ft. at ground speeds of less than 1 m.p.h. and at

TABLE XI

EFFECTIVENESS OF 1.2 PER CENT 2,4-D FOR SINKING HYACINTHS WHEN THE SPRAYS WERE APPLIED DURING THE WINTER WITH A BEAN JUNIOR DUPLEX SPRAYER

Date sprayed, 1949*	Minimum temperature when spraying, °F.	Lb. of 2,4-D per acre	% Sunk by April 1, 1949
Jan. 6	50	10	100
Jan. 7	61	10	100
Jan. 7	61	10	100
Jan. 7	63	5	90
Jan. 14	80	5	90
Feb. 1	32**	10	75
Feb. 1	32**	5	0

* Killing frosts occurred December 31, 1948, and January 1, 1949.
** Ice formed on foliage of plants.

delivery rates of 125 to 200 gal. per acre. Although much less efficient than a boom sprayer, it was possible to kill most hyacinths within the effective range of the Bean Junior Duplex sprayer (Table XI). The lesser efficiency of this sprayer as compared with the boom sprayer may be judged from the fact that it required the delivery of at least 50 more gallons of spray solution per acre to obtain equivalent coverage of the hyacinth foliage (125 vs. 75 gal. or less). Much of this extra spray solution was wasted as excessive run-off since uniform coverage was not possible, and the remainder of the waste was in the form of spray drift which from a 1.0 per cent solution was not effective for killing hyacinths.

In the initial tests with the Bean Junior Duplex sprayer an attempt was made to obtain adequate coverage of hyacinths at a delivery rate of 100 gal. per acre. This rate of delivery was approached when the spraying was done from non-obstructed banks, but not in the case of spraying an irregular fringe growth of hyacinths from a boat. For example, in one of the tests

which involved treating 29 acres of a fringe growth of hyacinths, 3,300 gal. of spray solution were required or an average of 149 gal. per acre.

Sprays applied during January caused sinking of all hyacinths when 2,4-D was applied at the rate of 10 lb. per acre but only 90 per cent of the plants sank after treatment with 5 lb. of 2,4-D per acre (Table XI). The lesser effectiveness of sprays applied to hyacinths with frost-damaged leaves (Table XI) is attributed mainly to a reduced transport of applied 2,4-D. Presumably a lethal dose of 2,4-D was not transported from the surface of the leaves into the growing tip of the rhizome. Results of greenhouse tests gave support to this view. For example, one set of hyacinths grown in the greenhouse was subjected to $-5°$ C. for 8 hours on each of two successive days and transferred to the greenhouse during the intervening period of 16 hours. After the plants had thawed at the end of the second low temperature period, they were sprayed with 2,4-D. In one case the entire browned aerial portion and in another case only the leaves were sprayed. When the leaves, but not the tip of the rhizome, were sprayed, the plants recovered in four days by sending out new leaves from the rhizome, as in the case of similar plants not sprayed. Spraying the tip of the rhizome which was held above the water surface, as well as all leaves, resulted in death of the plants in 11 days.

Results similar to those just described were obtained with ice-coated hyacinths transferred from a pond to the greenhouse (Yonkers, N. Y.) after two days of freezing temperatures under natural conditions in late November. In this case the frost damage was more severe, killing all foliage and roots, and causing them to turn black. The frozen plants recovered in 13 days by sending out new leaves from the tip of the rhizome. The frozen plants which were sprayed with 2,4-D recovered also since in this case the growing tip of the rhizome was one inch under water where it could not be contacted by the spray. These results show not only that hyacinths can tolerate freezing temperatures for 48 hours but also that applied 2,4-D is not transported in a lethal dose from frost-damaged leaves into the growing point of the rhizome. Thus it appears unlikely that a 2,4-D spray applied to hyacinths immediately after a killing frost would contact the rhizome tips of a sufficient percentage of the plants to constitute an effective treatment. The 2,4-D spray treatment should be delayed until a substantial amount of regrowth is seen, which would require a week or longer depending upon the severity of the frost damage and weather conditions thereafter.

Tests with a gun nozzle equipped with a 16/64 inch tip and operated by a Bean Royal 35 pump were started in June, 1949. The effective range of this sprayer varied from 15 ft. at a speed of 3 m.p.h. to about 50 ft. at 1 m.p.h. The relation between concentration of 2,4-D, rate of application, rate of delivery, and the speed of spraying are shown in Table XII. The

approximate minimum requirements for effective spraying with the Bean Royal 35 sprayer were 0.6 per cent 2,4-D, a delivery rate of 150 to 200 gal. per acre, an application of about 8 lb. 2,4-D per acre, and a spraying distance ranging from 15 ft. at a speed of 3 m.p.h. to about 50 ft. at a speed of 1 m.p.h. In these tests (Table XII) a 0.12 per cent solution of 2,4-D was ineffective for eradicating hyacinths. Respraying with 0.12 per cent 2,4-D after 56 days was also ineffective.

Attempting to secure effective sinking of hyacinths by applying low concentrations (0.12 to 0.3 per cent) at delivery rates substantially higher than 200 gal. per acre were not successful since only about 25 per cent of

TABLE XII

RELATIONSHIPS BETWEEN CONCENTRATION OF 2,4-D, RATE OF APPLICATION, RATE OF DELIVERY, AND SPEED OF SPRAYING WHEN THE SPRAYS WERE APPLIED TO HYACINTHS WITH A TRIGGER TYPE BEAN GUN (16/64 TIP) OPERATED BY A BEAN ROYAL 35 PUMP ADJUSTED TO DELIVER 15 G.P.M. AT A PRESSURE OF 150 LB. P.S.I.

Acres treated	Concn., %	Gal. per acre	Lb. per acre	Spraying speed, m.p.h.	Percentage of plants sunk after days indicated			Percentage regrowth in 112 days
					80	98	112	
1.3	1.2	154	15	1.0	20	90	100	0
0.8	1.2	122	12	1.1	10	25	85	0
0.5	0.6	196	10	0.9	80	90	100	0
1.3	0.6	154	8	1.0	20	40	100	0
0.5	0.3	266	7	0.5	5	15	25	0
1.0	0.3	200	5	0.7	5	10	25	0
1.05	0.12	300	3	0.7	10	10	20	25

the plants sank in 112 days (Table XII). The limitation in this case would appear to be the quantity of 2,4-D deposited on the hyacinths. For example, when 5 lb. of 2,4-D were applied in 200 gal. of water to one acre of hyacinths, the actual quantity of 2,4-D retained by the foliage would be less than 5 lb. per acre by the amount in the run-off solution which is considered a total loss. Increasing the rate of delivery to 266 gal. per acre would increase the quantity of solution lost by run-off without increasing the deposit of 2,4-D left on the foliage. Similar results were obtained with a four-nozzle boom in which case 57 gal. of spray solution per acre gave adequate coverage of hyacinths growing in the experimental pits (1, p. 381). The percentage of plants sunk was not increased by the use of 70 gal. of the same concentration of 2,4-D.

In June, 1949, the first tests were carried out in which a new type of off-center one and one-quarter inch Teejet nozzle was used, which is referred to hereafter as a TOC jet. By using nozzle tips with different orifice sizes the rate of delivery could be varied from about 15 to 175 gal. per acre when the nozzle was held at a height of 4 ft. The effective spraying range varied from 8 ft. for the smallest size tip (No. 20) to 30 ft. for the

large-size tip (No. 150) when operated with a Porto Pump equipped with an adjustable valve for regulating the output according to the nozzle requirements and at a ground speed of 3 m.p.h. (Table XIII). The largest size tip (No. 300) furnished by the manufacturer was not used.

The TOC jet No. 150 delivered a flat spray which was equivalent in coverage to that delivered by a conventional boom containing from 20 to 25 one-fourth inch Teejet nozzles. With TOC jet No. 150 uniform killing of hyacinths over a distance of 30 ft. was accomplished by delivering 8 lb.

TABLE XIII

EFFECTIVE SPRAYING RANGE OF TOC JETS WHEN OPERATED BY A PORTO PUMP WITH THE BY-PASS VALVE SET AT 75 LB. P.S.I. AND THE SPRAYER MOVED AT A SPEED OF 3 M.P.H.

No. of TOC jet	Output, g.p.m.	2,4-D concn., %	Lb. per acre	Distance effectively sprayed, ft.	Marginal drift	Percentage of hyacinths killed after 42 days
150	12	0.36	3	20	None	50
		0.7	5	25	None	100
		1.4	8	30	None	100
80	7	1.4	9	15	None	100
		2.4	15	15	None	100
40	2.5	1.4	5	10	Moderate	100
		3.6	12	10	Slight	100
20	1.5	1.4	4	8	Pronounced	90
		3.6	9	8	None	100

2,4-D in 75 gal. of solution per acre at a spraying speed of 3 m.p.h. (Table XIII). Equivalent killing over a distance of 25 ft. by means of spray delivered from a Bean Junior Duplex sprayer required the delivery of 8 lb. of 2,4-D in 150 to 200 gal. of solution per acre at a pressure of 400 lb. p.s.i. and at a spraying speed of about 0.5 m.p.h. When the Bean Royal 35 sprayer delivered 8 lb. of 2,4-D in 150 to 200 gal. of solution per acre at a pressure of 150 lb. p.s.i. and a spraying speed of 1 m.p.h., hyacinths were killed over a distance of about 50 ft. These results show that a TOC jet is considerably more efficient than a gun-type sprayer.

For any type of sprayer the effective range for killing hyacinths is determined by the dose of 2,4-D as well as by the output in g.p.m. for a given nozzle. For example, when TOC jet No. 150 with an output of 12 g.p.m. was moved at a speed of 3 m.p.h., the effective range was reduced from 30 ft. to less than 20 ft. if the quantity of 2,4-D was decreased from 8 lb. to 3 lb. per acre (Table XIII). At a spraying distance of 20 ft., 3 lb. per acre of 2,4-D killed only 50 per cent of the hyacinths and consequently the treatment was not effective.

Since several different types of spray nozzles were used, it was necessary to calibrate each one so as to determine the proper output requirements for delivering the type of spray desired. The following formula proved useful in checking changes made in spraying equipment and in designing tests which involved the variation of one or more of the factors represented:

$$\text{g.p.m.} = \frac{\text{gal. per acre} \times \text{width of sprayed area in ft.} \times \text{speed in m.p.h.}}{495}.$$

The constant 495 is the time in minutes required to spray an area of one acre, in the form of a strip one foot in width, at the rate of 1 m.p.h. Best results were generally obtained with spray equipment which operated at pressures of 150 lb. p.s.i. or less. Higher pressures caused excessive spray drift and a waste of solution. Spray drift or an atomized spray was not effective for killing hyacinths unless applied in relatively high concentrations, for example, 20 per cent or higher.

Results obtained in a large borrow pit. In April, 1949, the lower borrow pit had a fringe growth of hyacinth and alligator weed about 30 ft. wide and 7 to 8 miles long with a total area of about 29 acres. Alligator weed occurring in scattered patches occupied about a fifth of the total fringe area, being concentrated particularly in shallow water around the entrance of two canals from which most of the alligator weed had come, and also at the south end. The initial treatment in April consisted of applying a 1.2 per cent solution of the 2,4-D amine salt at the rate of about 150 gal. per acre with boat-mounted equipment. This, together with follow-up patrol maintenance sprays applied in June, resulted in sinking all plants of both species by July 28 (5, p. 77). The faster sinking of alligator weed in the borrow pit as compared with its slower sinking in the experimental pits is attributed to the greater disturbance by wind and water currents and the greater depth of water in the borrow pit. However, at the time of the second treatment the alligator weed constituted 95 per cent of the floating material, so that it sank at a slower rate than the hyacinths.

Eradication of alligator weed in small canals. Several small canals ran through the swamp lying between the upper and lower guide levees of the Spillway area. Two of these canals leading into the north end of the lower borrow pit were covered with a rank growth of alligator weed which was the main source of supply of this species for the infestation of the borrow pit previously mentioned. These canals were 50 to 100 ft. in width with a narrow channel 6 to 8 ft. deep running through the center. In many places high mud shelves extended from the canal banks to the edge of the channel where they dropped off sharply. The shelves were covered with alligator weed anchored for the most part directly in the mud but in some cases under a layer of water varying in depth

from an inch to more than a foot. In other places the floor of the canal sloped gradually away from the outer margin without ending in vertical banks along the central channel. Although the alligator weed extended across the central channel in many places, the submerged stems were not dense enough or sufficiently anchored to prevent the passage of a rowboat.

The first treatment of 1.0 per cent 2,4-D applied from a boat caused killing of the tops of alligator weed throughout most of the sprayed area. Mats of alligator weed extending into the channel from the high mud shelves were first to become dislodged, and these were followed by mats breaking away from the main banks. The dislodged mats either sank in the deep water of the channel or were carried away by wind and water currents. Groups of submerged stems also broke away from the main mass as a result of wind and current action on stems made brittle by the 2,4-D treatment. Plants anchored to the bottom in shallow water grew again from the submerged stems or from stems which became exposed after a drop in water level during the dry season. Much of the alligator weed which grew from stems anchored originally under shallow water was left stranded on the exposed mud surface when the water level dropped several feet. This type of growth was readily killed by the second application of 2,4-D. A third application of 2,4-D was necessary to cause sinking of the small amount of floating material which was caught in snags along the main banks of the canal. Clearing of these two canals required six months and the use of three sprays. It is doubtful whether this could have been accomplished without the aid of a substantial drop in water level after the initial spray was applied. In fact, the success of practical control operations in many cases depended upon the proper correlation of chemical treatments with changes in water level in the borrow pits and canals.

CALIBRATION OF THE HELICOPTER

In order to determine the width effectively covered by spray delivered from the Bell 47-D type helicopter, tests were carried out in which the 40 per cent 2,4-D amine salt was sprayed from altitudes of 10 to 80 ft. at an air speed of 30 m.p.h. The helicopter boom was equipped with 54 nozzles for this series of tests. The approximate effective killing ranges were as follows: 60 ft. at a 10-ft. flying height, 80 ft. at a 20-ft. flying height, 110 ft. at a 40-ft. flying height, and 140 ft. at an 80-ft. flying height. Killing of the foliage of alligator weed was used as the criterion of the width effectively covered (5, p. 82). On the basis of these results calibration curves were constructed to show the relation between width effectively covered, rate of applied 2,4-D per acre, and the number of nozzles needed for delivering 2 gal. of spray solution per acre (Fig. 7 A and B). For example, if the spraying width is 140 ft., the full quota of 82 nozzles would be required to deliver 8 lb. 2,4-D per acre (Fig. 7 B). If the spraying width is 70 ft., the

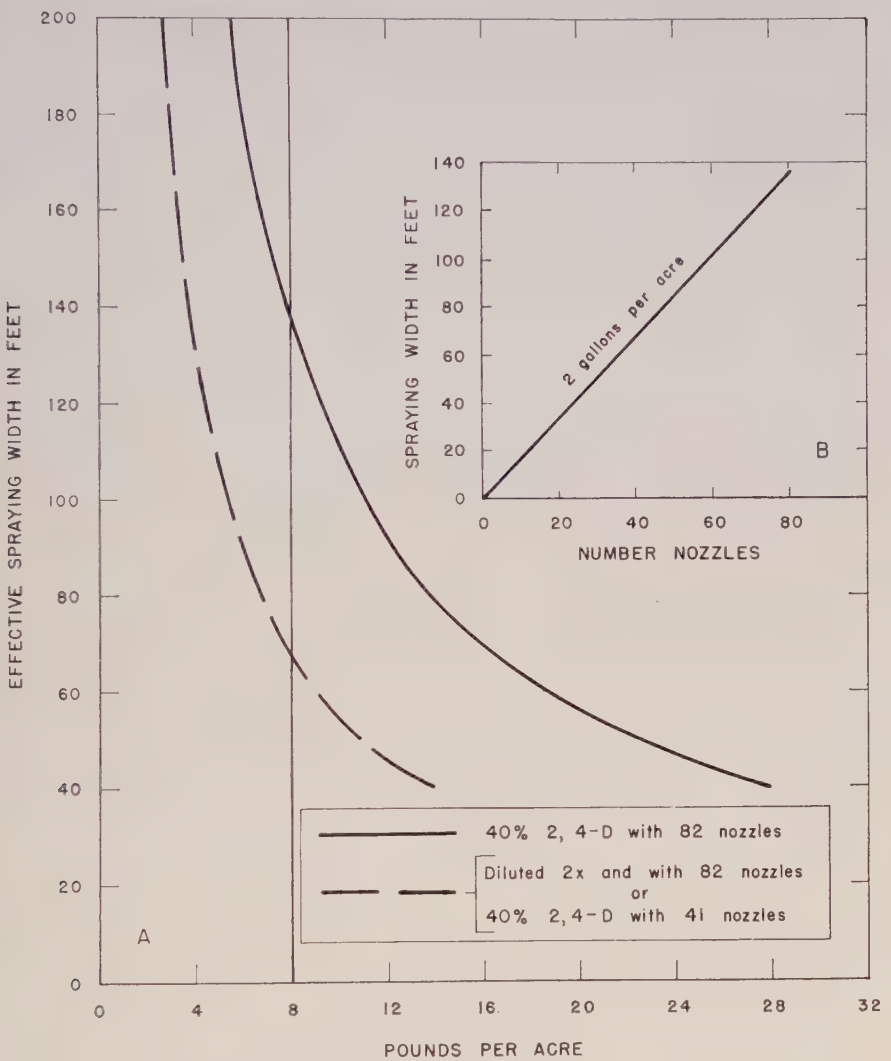


FIGURE 7. A. and B. Calibration curves showing number of Whirljet ¼ BAL No. 1 nozzles, each delivering 0.20 g.p.m., required to apply a given quantity of 2,4-D on a given width when the helicopter delivered 2 gal. of spray solution per acre at an air speed of 30 m.p.h.

requirements would be 42 nozzles and a flying height of 15 ft. in order to deliver 8 lb. of 2,4-D per acre. In this case the use of 82 instead of 42 nozzles would result in twice the rate of application (15.6 lb. per acre) and consequently a waste of about 8 lb. of 2,4-D per acre. Conversely, attempting to spray a width of 70 ft. by flying at an altitude of 80 ft. with the use of 42 nozzles would result in the application of 4 lb. of 2,4-D per acre.

Although 20 per cent 2,4-D was effective for killing hyacinths, no conditions were encountered which would justify carrying the extra water in 20 per cent 2,4-D. Practical control of hyacinths and alligator weed by means of 2,4-D applied with a helicopter is dependent upon the quantity of 2,4-D applied per acre and not the concentration of the chemical. Thus it is more economical to carry 25 gal. of 40 per cent 2,4-D since twice the area can be covered or the same area can be covered in half the time as compared with using 20 per cent 2,4-D (Fig. 7). It is to be noted that 70 ft. is the maximum width which can be covered with an 8 lb. per acre dose of 2,4-D when applied as a 20 per cent solution (Fig. 7).

Measurements for the effective spraying widths shown in Figure 7 for killing foliage of alligator weed approximate those obtained by Roth of the Bell Aircraft Corp. for killing insects (3), namely, at an air speed of 30 m.p.h. the effective spraying width was 100 ft. at altitudes of 20 to 25 ft., a 60 ft. coverage at an altitude of 10 to 12 ft., and a 45 ft. coverage at altitudes of 0 to 5 ft. The mass average diameter of the droplets produced by the whirl jet B-1 nozzle was about 250 microns. No difficulty in spraying in cold weather was encountered when the viscosity of the spray solution had a Saybolt reading less than 70 to 75 seconds at 100° F. Other pertinent suggestions made by Roth are that in spraying close to trees a reduction in forward speed would give greater downwash and more lateral roll into the overhanging tree boundaries, that placing of nozzles at right angles to the air flow gives more uniform distribution of the spray when 40 nozzles or less are used, and that less than 20 nozzles should be located on the outside two-thirds of the boom, leaving the first four bosses closest to the tail assembly without nozzles. These suggestions apply to the use of half the total number of nozzles on each of the two boom sections located on either side of the tail assembly. Another suggested method of reducing the number of nozzles by one-half is to use only one of the two boom sections, cutting out the other section by a shut-off valve located between the boom section and the solenoid valve.

RESULTS WITH HELICOPTER SPRAYING

For the tests carried out in July, 1949, the helicopter boom was equipped with 66 nozzles, each delivering 0.2 g.p.m. at a pressure of 45 lb. p.s.i. or a total output of 13.2 g.p.m. The spraying speed was 30 m.p.h. and the flying height varied in different tests from 10 to 80 ft. Triethanolamine salt of 2,4-D was used at a concentration of 20 to 40 per cent and applied at an estimated 2 gal. per acre. Both hyacinth and alligator weed were included in the tests.

Test 1. The upper borrow pit was 3.5 miles long, 400 to 500 ft. wide, and up to 50 ft. deep with a total area of about 125 acres, which was covered with a densely-packed growth of hyacinths (Fig. 8 A). Ten loads

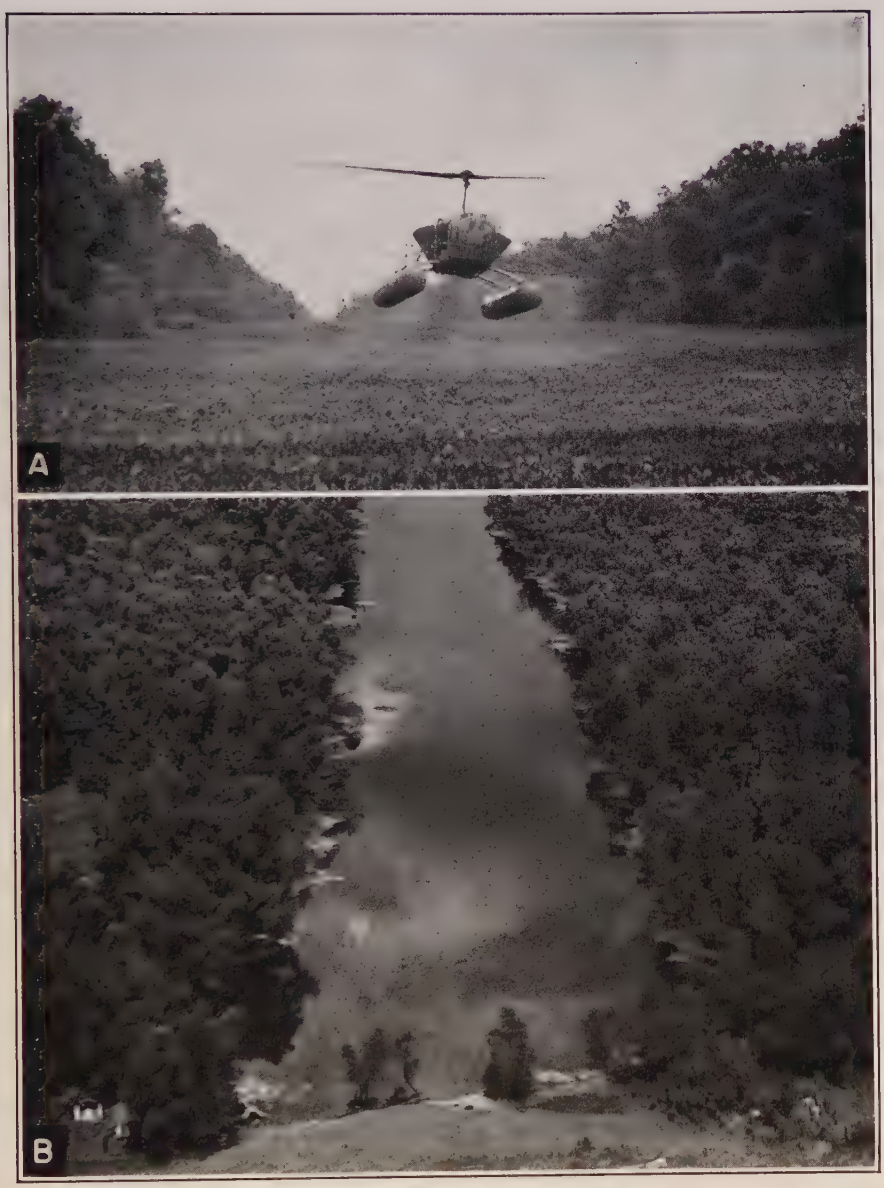


FIGURE 8. A. Hyacinths in upper borrow pit located on the Bonnet Carré Spillway Reservation being sprayed with 40 per cent 2,4-D from a Bell 47-D type helicopter July 19, 1949. B. Air view of same area of the upper borrow pit taken seven weeks later showing 90 per cent of the area cleared and only a narrow remaining fringe composed of hyacinths not contacted by a lethal dose of the spray and dead plants which had not yet sunk.

of 25 gal. each of 40 per cent 2,4-D were applied from a height of 10 ft. in 2.5 hours. No attempt was made to treat hyacinths along the irregular margins which, as was learned later, would necessitate flying above the trees that overhung the banks. When flying at a speed of 30 m.p.h. close to the trees along the banks, there was not sufficient lateral roll of the delivered spray to move in under the trees, so a strip of hyacinths along each bank was not treated with a lethal dose of 2,4-D. Additional hyacinths escaped being treated with a lethal dose as a result of the successive routes by the helicopter not being close enough together. As was learned in later tests, a flying height of 30 to 40 ft. would have given better and more efficient coverage. Intermittent showers occurred through the course of the test and during the remainder of the day.

Uniform browning of the hyacinth foliage occurred in 30 minutes when no rain fell in that period. The discoloration was delayed as much as 24 hours when rain fell in 5 to 15 minutes after treatment. In the case of an operation lasting all day, browning of the hyacinth foliage could be used as a guide by the pilot for determining the area adequately covered, provided rain did not fall within 30 minutes after treatment. In spite of the areas of hyacinths which appeared not to have been adequately covered and the interference caused by intermittent showers, the treatment resulted in the sinking of about 90 per cent of the hyacinths (Fig. 8 B). The final patrol maintenance spray applied with boat-mounted equipment in November resulted in the sinking of all remaining hyacinths in the borrow pit.

Test 2. A section of the upper guide levee canal, 0.8 mile in length, where trees lined both banks, was selected for determining the relative effectiveness of applying a 40 per cent 2,4-D spray from above the tops of the overhanging trees which, in this case, was about 80 ft. The average width of the canal was 60 ft. and the depth 6 to 8 ft. Heavy rain fell ten minutes after the spray was applied.

Complete killing and sinking of all hyacinths in the treated area by October 30 showed that the 2,4-D spray falling through the opening between the two rows of trees settled downward and also outward through the foliage of the trees with the result that all hyacinths from bank to bank were thoroughly covered. This is one of the few cases in which all plants were killed by the first spray so that a second spray was not needed. Foliage of willow (*Salix* sp.), poplar (*Populus* sp.), sycamore (*Platanus* sp.), and cypress (*Taxodium distichum* [L.] Richard.) was badly damaged or killed on the canal side of the trees, but not on the opposite side. Cypress developed a new crop of leaves one month later. There was no noticeable damage to foliage at a distance of 25 ft. or more from the banks of the canal.

Test 3. A borrow pit having an area of 21 acres and a depth of 40 to 50 ft. had been covered with a dense growth of hyacinths for four years with

the result that a flotant had formed which was 4 to 5 inches in thickness. A 20 per cent solution of 2,4-D was applied at a height of 10 ft. during a period of intermittent showers. The hyacinths required a much longer time to sink than in the previous two tests. Three factors operated against the sinking of hyacinths within three months, namely, a lower concentration of 2,4-D, intermittent showers, and the presence of a hyacinth flotant. The final spray applied in less than half a day from a small boat on November 28, resulted in the sinking of all hyacinths by the next observational date on January 13, 1950.

Test 4. The lower guide levee canal was 3.5 miles long, 60 to 75 ft. wide, and 6 to 10 ft. deep. Tall trees up to 100 ft. in height lined the swamp side of the canal and short trees 50 ft. or less in height, mostly willows, were widely scattered along the opposite levee bank. The canal was covered with a dense growth of hyacinths except at the south end where a solid stand of alligator weed occupied a distance of about 500 ft. One-third of the length of the canal was sprayed with 40 per cent and the remainder with 20 per cent 2,4-D applied from a height of 40 to 80 ft. The helicopter flew off center toward the levee bank so that equal coverage was not attained along the opposite, swamp side of the canal.

Reduction in the rate of killing and sinking resulted from a sudden change in flying height from 40 to about 80 ft., from lowering the concentration of 2,4-D from 40 to 20 per cent, and from rain falling ten minutes after treatment. Regardless of these adverse effects on the rate of sinking, most of the hyacinths and also the alligator weed eventually sank. The few remaining plants were eradicated by a second treatment with 1.0 per cent 2,4-D applied November 28 with a Hudson Junior sprayer, operated from a small boat. This operation required less than half a day. The canal was completely clear by the next observation date, January 13, 1950.

Test 5. Long Island Bayou was about 5 miles long, 50 to 75 ft. wide, and relatively shallow, being generally less than 5 ft. deep but deeper at the upper end than at the lower end. Alligator weed covered the entire width of the bayou in many places and occurred as a fringe growth in other places. In the deeper portion, the alligator weed was anchored mainly to the banks, whereas in the more shallow portion plants were anchored to the bottom as well as to the banks. Forty per cent 2,4-D was applied at a flying height of about 10 ft., a total of 120 gal. being used for this operation. The rate of application was estimated to be about 10 lb. of 2,4-D per acre.

Within one month after treatment, all of the alligator weed in the upper deeper portion of the bayou became dislodged from its anchorage and either sank or was carried out by tidal currents into the Gulf of Mexico. Although the tops of alligator weed were killed to water level in the lower portion of the bayou, there was no substantial reduction of the submerged mass of

stems that was anchored to the bottom. New shoots grew from the submerged stems at a slow but steady rate until at the end of nine months the growth in the shallow end of the bayou was about the same as it was originally. A second spray of 2,4-D was not applied to this area.

Test 6. Alligator weed growing in the Delta marshes represents a special type of growth not found on the mainland. Periodic deposition of silt had buried the stems of alligator weed to a depth of a foot or more, so that when the plants were killed to ground level by an application of 2,4-D, a

TABLE XIV

RELATIVE EFFECTIVENESS OF DIFFERENT CONCENTRATIONS OF THE 2,4-D AMINE SALT WHEN APPLIED BY HELICOPTER TO HYACINTHS GROWING IN A CANAL. RESULTS EXPRESSED AS THE PERCENTAGE OF PLANTS SUNK IN 76 DAYS

Concn.,* %	Estimated lb. per acre	Effective coverage bank to bank, %	Plants sunk, %
40	8	99	100
20	4	95	98
10	2	85	10
5	1	85	5

* Diluted on volume basis.

vigorous regrowth from the buried stocks occurred in one month after treatment. It seems unlikely that this type of growth can be eradicated by means of 2,4-D sprays.

Test 7. A tree-lined portion of the canal along the Airline highway was selected for applying 5, 10, 20, and 40 per cent 2,4-D by helicopter to a bank-to-bank growth of hyacinths. The width of the canal was 60 to 80 ft. and the depth 8 to 10 ft. Each concentration of 2,4-D was applied to a section 0.5 mile long from a height of 30 ft. The flight was made down the center of the canal which resulted in the spray covering a minimum width of 100 ft. Forty per cent 2,4-D caused sinking of all hyacinths. These results are comparable to those obtained in Test 2 where a single treatment caused sinking of all hyacinths when the helicopter was flown over the tree tops at a height of 80 ft. The 20 per cent application of 2,4-D caused sinking of all except a few hyacinths located underneath the overhanging trees, but the lower concentrations of 5 and 10 per cent caused only a small amount of sinking (Table XIV) with the result that regrowth occurred throughout the treated area. Hyacinths treated with 40 per cent 2,4-D sank at a faster rate than those treated with 20 per cent.

HYACINTH FLOTANTS

When hyacinths become tightly packed in a confined area where no lateral movement can occur, a matted layer one inch or more in thickness

is formed from old leaves and roots. This type of growth in deep water is here referred to as a hyacinth flotant and is to be distinguished from a similar growth in shallow water where the roots of hyacinths grow into solid matter on the floor of the waterway with the result that the water under the matted growth is not deep enough for the submergence of the mat. Hyacinth flotants up to about five inches in thickness were observed under natural conditions. In one case an area of 21 acres of water 50 ft. in depth had been covered with a jammed growth of hyacinths for four years. The thickness of this flotant was four to five inches. Thin flotants were formed in the experimental pits where the hyacinths remained undisturbed for six months or longer in a densely-packed mass. The term "flotant" is used in a modified sense to apply to floating hyacinth mats, and not in a general ecological sense as used by Penfound and Earle (2).

Sinking of a hyacinth flotant was accomplished by treating the plants with an effective dose of 2,4-D as in the case of hyacinths which grew under conditions in which lateral movement could occur. However, there was a considerable difference in the time for all hyacinths to sink, the flotant growth requiring about 40 days longer. Sinking of a treated hyacinth flotant was hastened by mechanical disturbance of the water such as occurred with the passage of a boat through the area.

In some cases, as, for example, in Lake Labranche, the water was deep in the center and shallow at the margins and for a considerable distance inward from the banks. The entire surface of the water except for the deeper central area was covered with hyacinths. Treatment of the entire lake area with 2,4-D caused sinking of the flotant in deep water before the dead mass of marginal plants had disappeared. The marginal mat of dead hyacinths eventually disappeared by a successive breaking away of the material from the inner edge since there was not a sufficient depth of water underneath for the dead material to sink in large masses. These results indicate that if a waterway is uniformly shallow enough for the roots of all or most of the hyacinths to grow into solid matter underneath, treatment with 2,4-D might not cause sinking of the dead plants. This type of growth is not to be confused with a marginal growth of hyacinths when all roots of the plants are growing in wet soil as a result of the plants being stranded when the water level declines. Hyacinths growing in soil were killed more readily than those with roots in water (1, p. 369).

Treatment of a hyacinth flotant in the experimental pits caused killing and disintegration of the foliage with the result that much of the surface of the flotant was exposed for a period of two to three weeks before sinking occurred. If the flotant remained exposed for three weeks or longer during the summer months, many seedling hyacinths appeared. Most of the seedlings were carried down with the flotant when it sank. The longer the surface of the flotant remained exposed, the greater the number of seedlings

which appeared and the greater the number which survived (Table XV). When 2,4-D was applied to the hyacinth flotants in experimental pits during the period May through July, seedlings appeared in 55 to 77 days after treatment. Since an 8 lb. per acre dose of 2,4-D caused the flotant to sink within three months (Fig. 1 B), relatively few seedlings appeared in this time and few or none survived (Table XV). Less effective treatments resulting from lower doses of 2,4-D or from interference by rain, permitted the survival of a substantial number of seedling hyacinths (Table XV).

When a flotant was not present at the time of treating the hyacinths,

TABLE XV

INFLUENCE OF HYACINTH FLOTANT FORMED IN PITS BEFORE TREATMENT ON SURVIVAL OF SEEDLINGS WHICH APPEARED 65 TO 70 DAYS AFTER MAY OR JUNE TREATMENTS THAT KILLED ALL OF THE ORIGINAL PLANTS, THUS EXPOSING THE SURFACE OF THE FLOTANT TO LIGHT AND HIGH TEMPERATURES

Area treated, sq. ft.	No. days after treatment flotant sank	No. days surface of flotant remained exposed	Maximum number of seedlings observed*	Number of seedlings surviving
3600	84	8–19	153	3
1800	96	20–40	180	69
1800	129	50–80	3800	391

* At any one count.

none was found as a result of applying 2,4-D to plants in the experimental pits or in the large waterways. Practically all navigable waterways and all important drainage canals in Louisiana are worked on or disturbed frequently enough to prevent the formation of flotants. Conditions favorable for the germination of hyacinth seed and for the growth of young seedlings seldom occur in nature (1) so that there is no substantial reinfestation from a seedling source. Even if seedling hyacinths germinated after an initial treatment with 2,4-D, they would be killed by a patrol maintenance spray. Young seedling hyacinths proved to be more sensitive to 2,4-D than mature plants, most of which developed as offshoots.

PRODUCTION OF SEEDLING HYACINTHS UNDER NATURAL CONDITIONS

The rapid spread of hyacinths throughout the waterways of Louisiana is mainly due to vegetative propagation by means of offshoots, even though seed formation reaches fantastic numbers each year. The fact that seedling hyacinths are seldom seen growing under natural conditions led to a study of the requirements for germination of the seed and for growth of the young seedlings. Results of greenhouse tests carried out from December, 1948, to February, 1949, showed that temperature was the principal limiting factor for germination of hyacinth seed after it had passed through a dormant period of about two months in water or in a moist medium (1).

Hyacinth seed in shallow water germinated in one to two weeks when the temperature of the water was 28° to 36° C. during the main part of the day and about 21° C. during the night. The seeds also germinated on a thin layer of water-saturated soil in a Petri dish in the greenhouse.

In view of the fact that hyacinth seed is heavier than water and much of the seed would sink to the bottom of the water in which the seed was discharged, it was of interest to know whether the seed could germinate under at least a foot of water as well as under water only a few inches in depth. Tests carried out in August, 1949, in the greenhouse showed that hyacinth seeds from the same collection previously used (November, 1948) gave 100 per cent germination in four days when covered with 16 inches of tap water in a clear glass cylinder. Lower percentages of germination were obtained in shallower water. On the basis of 25 seeds each, the percentage germination for seeds under 1, 4, 8, 12, and 16 inches of water was 40, 60, 72, 72, and 100 per cent respectively. The corresponding volumes of water were 26, 104, 208, 320, and 1185 ml. Water temperatures fluctuated each day from 20° to 25° C. at 8:30 a.m. to 44° to 47° C. during the period 11:30 a.m. to 4:00 p.m. Since the rate of germination increased with increasing volume and depth of water, it would appear that the favorable effect in the larger volumes of water was due to a lag in temperature decrease each night. Hyacinth seed under six inches of water in a brown glass bottle gave 28 per cent germination in three weeks. The reason for this difference is not understood since it was shown by Penfound and Earle (2) that scarified hyacinth seed germinated in darkness. The production of hyacinth seed during 1948 and 1949 was observed to occur under natural conditions on a tremendous scale, so that failure to produce seed, as in the case of alligator weed, cannot account for the scant production of seedlings in nature.

After germinating, the hyacinth seedlings showed little or no increase in size when floating on tap water with or without soil in the bottom of the container. If the young seedlings were transferred to water-saturated soil, they grew rapidly up to the stage where two or three float leaves were formed, and thereafter the growth was much better when the seedlings were transferred to water having soil underneath (1, 2). About 30 days in soil were required for a newly germinated seedling to reach the stage where it could grow as a typical floating plant at a much faster rate than in soil. At the end of about 60 days when the seedling was three to four inches in diameter, offshoot formation started and continued at a rapid rate, 50 offshoots being formed in 30 days.

Having determined the importance of a relatively high temperature for the germination of hyacinth seed and the importance of a soil medium in the growth cycle of the young seedling under greenhouse conditions, an attempt was made to determine to what extent these factors were

involved in limiting the production of seedling hyacinths under natural conditions. During 1948 not a single seedling hyacinth was observed in open water or at the margins of any fringe growth of mature plants in the borrow pits and canals in the Bonnet Carré Spillway reservation. Of the many thousands of hyacinths transferred from these areas to the experimental pits none appeared to have seedlings entrapped in the crowns or in the root systems. The only place where seedlings were observed was on an exposed part of a floating mat where the adjacent plant population was composed of many other species besides hyacinths. This growth was not typical of that found on the navigable waterways.

Temperature records were taken of the water at or near the surface in borrow pits and in canals during March through May, 1949. Under a typical compact growth of hyacinths the water temperatures were 17° to 21° C. when the air temperatures were 26° to 31° C. In open water the temperatures were 20° to 32° C. during the same period. The temperatures around the upper part of the root system or on the wet part of the hyacinth mat which is shaded by the dense foliage were apparently below the minimum requirements for the germination of hyacinth seed. In April, 1949, hyacinth seedlings in all stages of development up to about two inches in diameter were found along the wet banks of the experimental pits and up to 4 ft. from the edges wherever the soil was saturated with water. However, seedlings were not found in the main part of the pit. The water level in many of the pits was at ground level and had overflowed the banks as a result of frequent rains during April and due to the fact that the Mississippi River and the water table were high, as is normal for the time of year. Presumably hyacinth seed had been carried beyond the edges of the pit with organic debris when the pit overflowed. Numerous seedlings were also observed along the banks of the lower guide levee canal.

During May there was little rain and the water level in the pits and canals fell 1 to 2 ft. with the result that the seedling hyacinths along the banks were stranded in soil which soon dried out and became baked. All of the stranded seedlings died from lack of moisture and no new seedlings appeared thereafter in these areas. Although the temperatures of the soil surface throughout the summer were as high or higher than in the spring, a water-saturated condition did not last long enough to support the growth of any seedlings which may have started. This is in contrast to mature hyacinths and offshoots which lived much longer on the margins of waterways after the water level dropped a foot or more. Seedling hyacinths and offshoots of all stages of development growing in soil died much faster when treated with 2,4-D than the offshoots having roots in water. The young seedlings were killed within a week after being sprayed with 2,4-D.

All evidence to date indicates that the principal effect of direct sun-

light on germination of hyacinth seed is in raising the temperature of the moist or wet germinating medium to at least 28° to 30° C. There appears to be an abundant supply of viable hyacinth seed in and on the dead or partially decomposed parts of hyacinths which forms a mat under the dense foliage canopy. In each of several instances when the foliage canopy was cleared away by hand, hyacinth seedlings appeared in one to two weeks on the exposed wet surface of the mat. Seedlings also appeared on hyacinth flotants after a 2,4-D spray had killed the foliage and exposed the wet surface of the mat but had not caused sinking of the flotant within three months from the time of treatment either because the dose of 2,4-D was ineffective or because the flotant was in shallow water where it could not sink. Thus if the surface of a hyacinth flotant remained exposed for more than three weeks, numerous seedlings appeared and a substantial number survived (Table XV). If the flotant sank before the seedlings had developed to the stage where they could continue growth as a free floating plant, they sank with the flotant and did not rise to the surface (Table XV). These results emphasize the importance of an initial period of about one month's anchorage in a solid wet medium in the growth cycle of young seedling hyacinths. Under natural conditions the factors which prevent young seedling hyacinths from surviving appear to be more important than factors which prevent the germination of seed. In addition to the adverse effect of a lowering of the water level on survival of hyacinth seedlings, wild animal life such as fish and muskrats in and around the water destroyed young seedlings as evidenced by numerous mutilated specimens and the disappearance of many seedlings under observation in a confined area.

In contrast to the appearance of hyacinth seedlings in areas cleared by hand, none appeared in similar areas cleared by means of 2,4-D sprays. Since in most practical control operations a patrol maintenance 2,4-D spray would be used in addition to an initial spray, any seedling hyacinths which might appear after the first treatment would be easily killed by the second spray. Except for the special conditions under which 8 lb. per acre of 2,4-D did not cause sinking of all hyacinths within three months, the presence of a substantial number of seedling hyacinths on a floating mat of dead or partially killed mature hyacinths would be an indication that the initial treatment was not effective either because the quantity of 2,4-D which contacted the hyacinths was too low or because of interference by rain. Seale and Allison (4) reported that seedling hyacinths appeared on mats of dead plants after spraying with 1 lb. per acre of 2,4-D by means of an airplane. This quantity, however, was found to kill only 62 per cent of the plants and according to present standards would be an ineffective treatment.

SUMMARY AND CONCLUSIONS

Tests carried out in 1948 and 1949 showed the following results: an 8 lb. per acre dose of 2,4-D was effective throughout the year in causing water hyacinths to sink in two to three months; the esters of 2,4-D were of about equal effectiveness to the amine salt; 2,4-D was more effective than 2,4,5-T; the amine salt of 2,4,5-T was more effective than the isopropyl ester; hyacinths were killed more readily in August than in June; it was difficult in practical control operations to contact all hyacinths with one 2,4-D spray and a second spray had to be applied to surviving plants before any substantial reinfestation had occurred; and the scant production of seedling hyacinths under natural conditions plays no important part in the rapid spread of this species in Louisiana.

The rate at which hyacinths sank after treatment with an effective dose of 2,4-D depended upon the age of the plants growing in a given location. Hyacinths which had been tightly packed in a confined area for six months or longer formed a flotant consisting of a dead mat of decaying leaves and roots held about the bases of the living hyacinths. The mat increased to a maximum thickness of about 5 inches over a period of several years. Hyacinth flotants required 30 to 40 days longer to sink than a mat of plants which had remained in a tightly packed condition for three months or less. A 2 lb. per acre dose of 2,4-D did not cause sinking of hyacinth flotants. Fringe growths of hyacinths sank more quickly than any type of growth which covered the entire surface of the water. If a hyacinth flotant was disturbed as, for example, by the passage of boats, sinking of the dense material was hastened. This applied also to floating mats of dead or partially killed alligator weed.

Alligator weed exhibited little or no growth and could not survive for more than 21 months unless its roots were anchored in soil. Plants anchored in soil along the banks of deep bodies of water were observed not to grow out into water having a depth substantially over 5 ft. This type of growth was easily dislodged and sunk by treatment with 8 lb. per acre of the 2,4-D amine salt which also caused sinking of 95 to 100 per cent of the hyacinths. When alligator weed in experimental pits was treated with 2,4-D in September and October, the plants sank at a faster rate than when treated during the period March through August. Alligator weed which was subjected to wind action, water currents, and boat traffic in navigable waterways sank faster at all times of year after treatment with 2,4-D than similarly treated plants growing in experimental pits where there was little or no disturbance of the water, and where there was an opportunity for some of the submerged stems to become anchored in soil at the shallow end of the pit.

If the principal anchorage of alligator weed growing in shallow water

was in the bottom, the submerged stems were not sufficiently damaged by 2,4-D to prevent a substantial regrowth. However, if the water level receded sufficiently during the dry season to expose most of the stems, the plants were killed by a follow-up treatment with 2,4-D. Alligator weed growing in the marshes of the Mississippi River delta could not be controlled by 2,4-D since root stocks buried a foot or more under periodic silt deposits sent up a new crop of shoots a few weeks after the aerial parts of the plants were killed.

Effective control of hyacinths and alligator weed depended upon the proper use of spray equipment for a given set of conditions. Sprays delivered by low-pressure equipment (150 lb. p.s.i. or less) were more effective than sprays delivered at pressures above 150 lb. p.s.i. The decreasing order of efficiency for sprayers used in practical control operations was: the helicopter which delivered 2 gal. of spray solution per acre, the TOC $1\frac{1}{4}$-inch boomjet delivering 75 gal. or less per acre, and the gun-type sprayer delivering 150 to 200 gal. per acre.

Conditions were not encountered in helicopter spraying where the use of concentrations of less than 40 per cent 2,4-D would justify carrying the extra water. Concentrations of 5 to 10 per cent 2,4-D were ineffective when delivered by helicopter since 2 gal. of the spray solution contained 1 lb. and 2 lb. of 2,4-D respectively, which is substantially below the minimum of about 8 lb. of 2,4-D per acre required for effective control of hyacinth and alligator weed under most conditions. Any width from 20 to 140 ft. could be adequately covered with 2 gal. of 40 per cent 2,4-D by varying the number of nozzles on the helicopter boom and the altitude of flying. An air speed of 30 m.p.h. and a flying height of about 40 ft. were considered most desirable for spraying large open areas of hyacinths or alligator weed with 2,4-D.

Seedling hyacinths appeared along the wet banks of the experimental pits, bayous, and canals in April when there were frequent rains and at the time of year when the Mississippi River and the water table were high. This wet period was followed by a dry period in May which was associated with a drop in water level of 1 to 2 ft. with the result that most of the young seedling hyacinths were left stranded in dry, baked soil where they died from lack of moisture. The high temperatures of water-saturated soil and shallow water exposed to direct sunlight were favorable for the germination of hyacinth seed. This is in contrast to the temperature of the water at or near the surface of hyacinth mats which was below the minimum (28° to 30° C.) required for the germination of hyacinth seed. When the foliage canopy was removed, hyacinth seeds on the exposed section of the mat germinated within one week. About 30 days were required for a young seedling to reach the stage where it could grow as a typical floating plant at a

much faster rate in water than in soil. The production of mature hyacinths from seed was limited by unfavorable growing conditions rather than by lack of conditions favorable for germination of the seed.

When chopped hyacinths and chopped alligator weed were thrown into the experimental pits, much of the material floated. There was abundant regrowth from this material in one month. If the chopped material was sprayed with 2,4-D, the segmented hyacinths sank in 23 days and the alligator weed in 57 days. These results indicate that when saw-boats are used to open channels through waterways infested with hyacinths and alligator weed, the chopped plants constitute an important source for reinfestation, but the material can be controlled with the same treatment used to control the intact plants.

Approximately 500 acres of waterways infested with hyacinths and alligator weed were cleared by applying 2,4-D at the rate of approximately 8 lb. per acre either by helicopter, by boat, or from the shore. The initial spray was followed by a second patrol maintenance spray to insure complete eradication.

ACKNOWLEDGMENT

The authors wish to acknowledge cooperation from the District Engineer, New Orleans District, Colonel Charles G. Holle, and staff. Especial thanks are due Colonel John R. Hardin, former District Engineer, and Mr. Walter C. Carey, Special Assistant to the District Engineer, who maintained an active interest throughout and made many helpful suggestions. Thanks are also due Mr. C. R. Dunn and Mr. C. Tomlinson, who gave valuable assistance throughout the project. Acknowledgment is due Dean Fred Cole who made the project possible through the facilities of Tulane University. All photographs were supplied by the Corps of Engineers, U. S. Army, New Orleans District.

LITERATURE CITED

1. HITCHCOCK, A. E., P. W. ZIMMERMAN, HENRY KIRKPATRICK, JR., and T. T. EARLE. Water hyacinth: its growth, reproduction, and practical control by 2,4-D. Contrib. Boyce Thompson Inst. **15**: 363–401. 1949.
2. PENFOUND, WM. T., and T. T. EARLE. The biology of the water hyacinth. Ecol. Monographs **18**: 447–472. 1948.
3. ROTH, ROGER W. [Helicopter spraying.] (Personal communication, Bell Aircraft Corp.)
4. SEALE, CHARLES C., and R. V. ALLISON. Hyacinth control through the use of 2,4-dichlorophenoxyacetic acid in different forms and at various rates of application. Soil Sci. Soc. of Florida. Proc. **8**(**1946**): 97–106. 1947.
5. ZIMMERMAN, P. W., A. E. HITCHCOCK, HENRY KIRKPATRICK JR., and T. T. EARLE. Practical control of water hyacinth with 2,4-D. Agric. Chem. **5**(2): 45–47, 49, 81, 83–85. Feb. 1950. (*Also in* Boyce Thompson Inst. Prof. Pap. **2**: 75–86. 1950).

MECHANISM OF PARTICLE SIZE EFFECTS OF FUNGICIDES ON PLANT PROTECTION

H. P. Burchfield and George L. McNew[1]

INTRODUCTION

The activity of 2,3-dichloro-1,4-naphthoquinone[2] as a foliage protectant against spores of *Alternaria solani* (Ell. & Mart.) Jones & Grout. is dependent upon particle size (17). On decreasing the mean radius from 24.5μ to 0.45μ only one-fortieth as much material is required to maintain equivalent blight control. Similar results have been reported for copper (7, 9, 20) and sulfur (5, 7, 21) fungicides; so the observation that finely divided agricultural preparations give superior performance can be accepted as a broad generalization. Qualitatively it is not difficult to see why this should be true. Highly fragmented dusts will provide spray deposits with large total surfaces from which the toxicant can readily solubilize and diffuse into adjoining areas of the foliage surface. Probably more important, a large number of particles, if distributed at random, will tend to provide uniform protection at all the possible loci of infection. Although these generalizations are useful in visualizing the conditions that prevail at the foliage surface, they do not provide a satisfactory framework against which to correlate the laboratory data, nor do they permit development of new concepts beyond the scope of the experimental data.

An examination of the dosage-response curves obtained with a series of Phygon samples with narrow particle size ranges indicated that the ED_{95} values were inversely proportional to the logarithm of the number of particles per unit area of leaf surface over the range studied. Furthermore, this relationship can be derived analytically if several basic assumptions are made concerning the mechanism of the particle size effect. By an extension of this treatment, some tentative conclusions can be made regarding the role of particle size in such diversified problems as persistence on foliage, treatment of seed, and the interpretation of dosage-response curves. Some of these effects are summarized in this report.

MATERIALS AND METHODS

Procedures for the determination of particle size distributions (6) and for the isolation of fractions with narrow particle size ranges (2) were the

[1] The writers are indebted to G. E. O'Brien and Delora K. Gullstrom for technical assistance and many valuable suggestions, and to the management of the Naugatuck Chemical Division, U. S. Rubber Co., where these investigations were made, for permission to publish these data.

[2] The active ingredient of Phygon, registered trademark. U. S. Patent 2,302,384 assigned to U. S. Rubber Co.

Copyright, 1950, by Boyce Thompson Institute for Plant Research, Inc.

same as those described in previous publications. The mean particle radii used in the calculations were determined by counting the number of particles per unit volume in a suspension of known concentration in a Levy blood counting chamber, and substituting in the equation $r = (3\bar{v}g/4\pi Nd)^{1/3}$ where r is the radius, N the number of particles, $\bar{v}$ the volume of suspension per square, g the grams of solid phase per ml., and d the density of Phygon. In some cases the fractions were the same as those described in the earlier paper (2); in others, new separations were made. The samples were quite homogenous. For example, the 3.57μ fraction contained 94.3 per cent by weight of the total material in the 3 to 5μ range as calculated from distribution equations. The scattering of material about the mean varied for each individual case, but can be computed readily from a knowledge of the range and method of separation.

Deposits of Phygon on foliage surfaces were determined by a modification of a colorimetric procedure previously described (3). Ten leaflets from each sprayed plant were removed and washed with three 10 ml. portions of acetone to dissolve the chemical. The combined extracts were placed in a 50 ml. volumetric flask and the plant pigments bleached out by a 5 to 10 minute exposure to direct sunlight. Five ml. of 10 per cent aqueous dimethylamine was then added and the contents of the flask made up to volume with additional solvent. Percentage transmission measurements were made at 490 mμ with a Lumetron colorimeter using as a blank a solution prepared from the leaves of an unsprayed plant. The weight of Phygon was calculated by substitution in the following equation: grams Phygon $= 0.05D/K$ where D is the optical density of the test solution, and K is the specific extinction coefficient obtained on a control sample of Phygon.

The leaflets were then blotted dry and pressed on an ink pad. Imprints were made on paper and the areas determined with a hatchet planimeter. In the calculations, the areas were doubled since it was assumed the deposits occurred uniformly on both sides of the leaves.

The photochemical decomposition of Phygon was studied by making up solutions in dioxane and acetone at concentrations of 5×10^{-3} mol./liter and permitting them to stand in direct sunlight. Ten ml. aliquots were withdrawn at intervals and made up to 25 ml. with dioxane, and then diluted to 50 ml. with a 0.1 N sodium tartrate buffer containing 0.05 per cent gelatin as a maximum suppressor. Measurements were made on a Sargent Model XX recording polarograph. The decomposition of the quinone was followed by measuring the drop in diffusion current with time, which, according to the Ilkovic equation (10, p. 30–39), is directly proportional to concentration.

The ability of Phygon to prevent infection of tomato foliage by *Alternaria solani* was tested by the method of McCallan and Wellman (15).

Seedling emergence tests were made by treating peas (*Pisum sativum*

L. var. Perfection) at doses of 0.25, 0.12, 0.06, 0.03, and 0.01 per cent by weight and tumbling the samples in quart jars on a vertical turntable for 30 minutes at 28 r.p.m. Eight replications of 25 seeds each were sown in randomized block arrangement in greenhouse flats filled with naturally infested composted soil. The seed was covered to a uniform depth, watered heavily, placed in a cabinet at 55° F. for seven days, and then removed to a greenhouse at 65° to 80° F. Records on emergence were taken 10 days later.

THE PROBLEM OF COVERAGE

In a previous publication (17), the ability of various particle size fractions of Phygon to protect tomato foliage against infection by *Alternaria solani* was illustrated by comparing ED95 values in parts per million in the spray suspensions with particle size ranges. While this demonstrated that the finely divided materials were more effective, it did not permit an

TABLE I

RELATIONSHIP OF CONCENTRATION OF SPRAY MIXTURE TO AMOUNT OF DICHLORONAPHTHOQUINONE DEPOSITED ON TOMATO FOLIAGE

Spray concn., p.p.m.	Total leaf area, cm.^{2}	Micrograms Phygon	Micrograms Phygon per cm.^{2}
960	160	880	5.5
	169	730	4.3
	191	820	4.3
	175	960	5.5
	153	870	5.7
	171	900	5.3
480	187	530	2.8
	171	450	2.8
	153	400	2.6

accurate analysis of conditions prevailing at the foliage surface. The actual amount of fungicide per unit area was unknown, and there was some question whether particles of different sizes would deposit and adhere at equivalent rates.

The first factor was evaluated by spraying tomato plants with 1 to 3μ material at concentrations of 480 and 960 p.p.m. and analyzing the main leaflets for residue. Results in Table I show that the deposits were directly proportional to concentration. It was found that 1 p.p.m. of fungicide in a spray suspension was equivalent to a deposition of 5.40×10^{-3} μg./cm.^{2} on the foliage surface with a standard deviation of 0.51×10^{-3} μg./cm.^{2}

Tests made on 1 to 3μ and 5 to 10μ samples sprayed at concentrations of 480 p.p.m. on filter paper targets showed no significant differences with respect to deposition between the two size classes. Microscopic examination and particle counts on effluent and residual suspensions obtained after

spraying with a DeVilbiss gun demonstrated that fractionation of the material or comminution of the larger particles did not occur.

On multiplying the ED95 values given in the preceding paper (17) by the deposition coefficient, they are translated into terms of μg. of chemical per square centimeter of leaf area. In Table II they are compared to the

TABLE II

RELATIONSHIP OF PARTICLE SIZE TO THE AMOUNT OF DICHLORONAPHTHOQUINONE REQUIRED FOR 95 PER CENT CONTROL OF EARLY BLIGHT ON TOMATOES

Av. radius of particles, microns	Amount required for ED95		
	Spray concn., p.p.m.	Deposit, μg./cm.²	No. of particles/cm.²
0.45	52	0.28	446,000
0.81	64	0.35	95,600
1.48	89	0.48	21,500
3.57	157	0.85	2,700
8.36	445	2.40	596
13.8	870	4.70	260
24.5	2,200	12.	118

mean particle radii computed for the various size fractions. If the particles are assumed to be approximately spherical, the number of particles per square centimeter leaf area can be computed from

$$N = \frac{3G}{4\pi r^3 d} \qquad (1)$$

where G is the ED95 in g. per cm.², r is the particle radius in cm., and d is the density of Phygon (1.645).

An interesting relationship exists between the amount of material per square centimeter and the number of particles necessary to maintain 95 per cent disease control with the various size fractions. The 24.5μ deposit must contain 40 times more material than the 0.45μ fraction, yet there are 4000 times as many of the smaller particles per unit of surface area. This situation can be appreciated more readily by reference to Figure 1 in which the protective particles and fungus spores have been drawn to scale at dosages corresponding to 95 per cent disease control. It is evident that the chances of direct contact with large particles are very small and that the fungicide must operate from a distance. By decreasing the particle size, the toxicant is made more available, and control can be maintained with a smaller amount of material. Assuming a random distribution, the availability must be a function of the number of particles into which the chemical is divided, and it is this conception that provides much of the basis for the following discussion.

On plotting ED95 values in p.p.m. against the logarithms of the radii (Fig. 2) a smooth curve results which shows that the effectiveness of

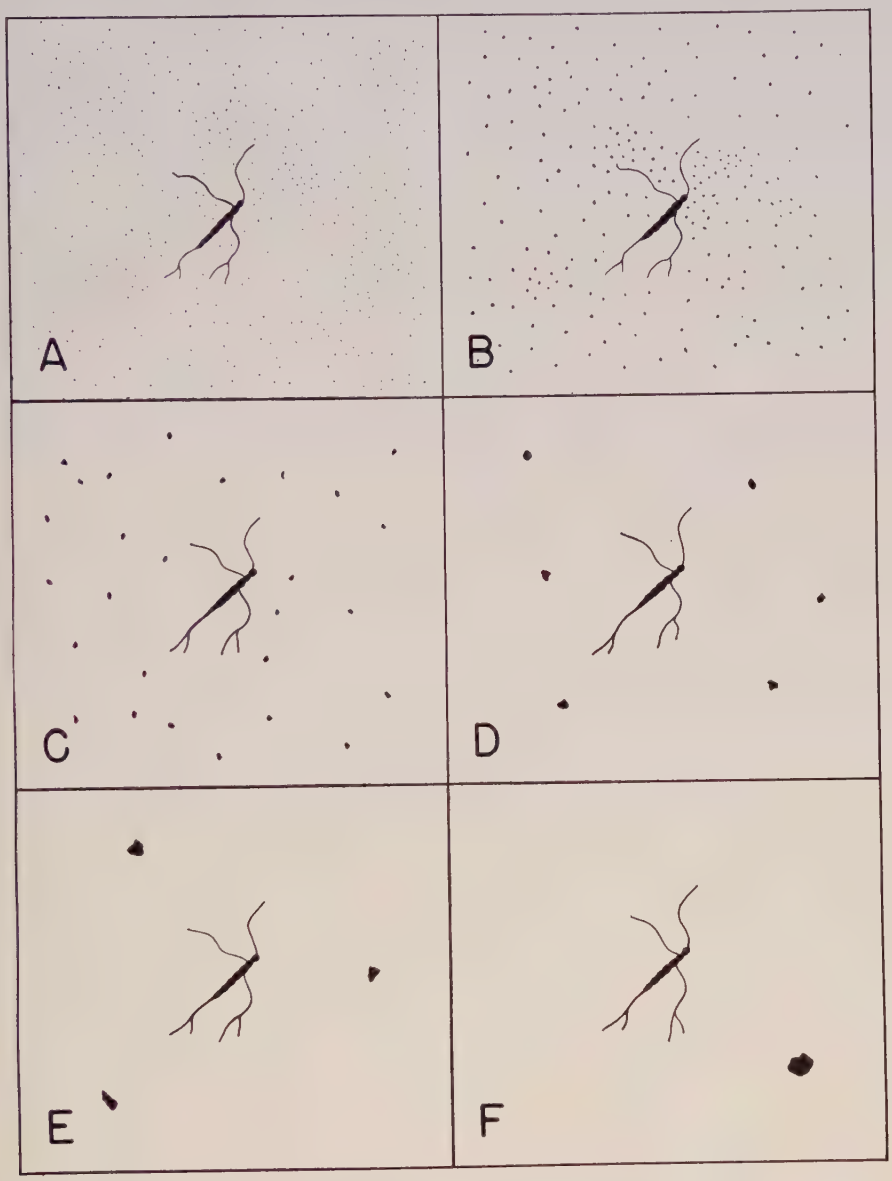


FIGURE 1. Representative relationship of a spore of *Alternaria solani* on .01 cm.^{2} of leaf area and deposits of dichloronaphthoquinone required to provide 95 per cent disease control by particles of radii 0.81 (A), 1.48 (B), 3.57 (C), 8.36 (D), 13.8 (E) and 24.5 (F) microns.

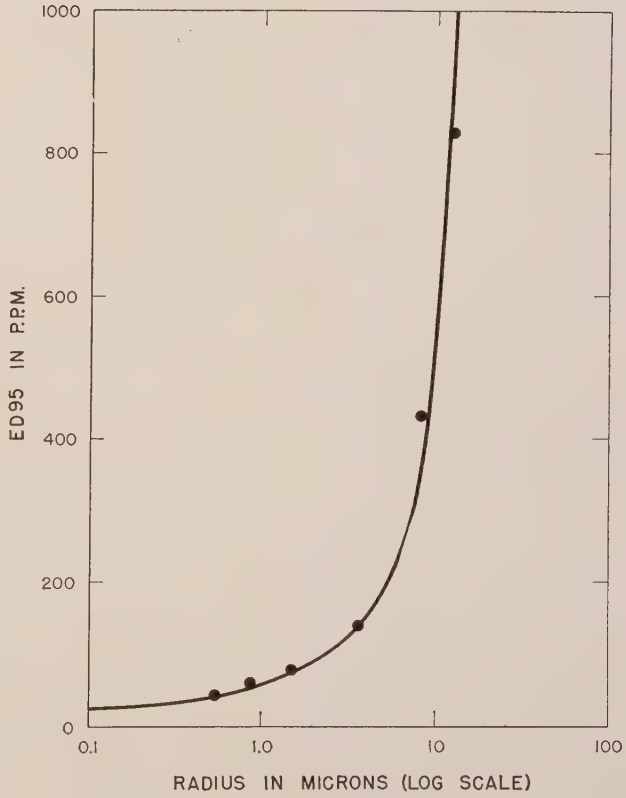


FIGURE 2. Relationship between the size of particles and concentration of dichloronaphthoquinone required to provide 95 per cent protection to leaves (equation 2).

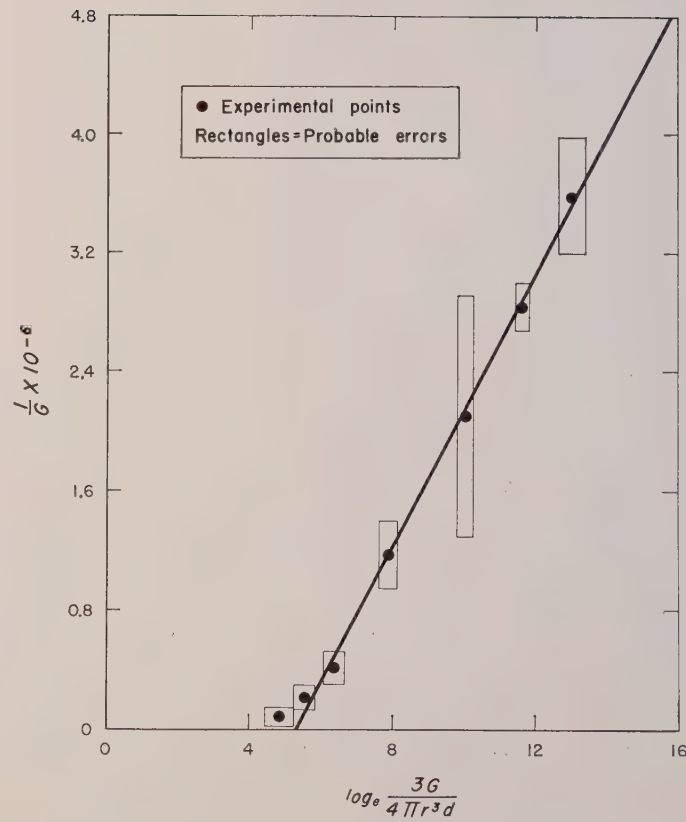


FIGURE 3. Relationship between reciprocal of dosage (G) and logarithm of number of particles at 95 per cent disease control (equation 2).

utilization increases quite rapidly on decreasing the particle size down to the 1 to 5μ region, but thereafter the rate drops off and appears to approach a limiting value.

This relationship can be expressed in a quantitative way by plotting reciprocals of the doses against logarithms of the number of particles (Fig. 3). A straight line is obtained which can be expressed by the equation

$$\frac{1}{G} = m \log_e \frac{3G}{4\pi r^3 d} + q. \qquad (2)$$

This equation states that the amount of material required to maintain 95 per cent disease control is inversely proportional to the logarithm of the number of particles into which it is subdivided.

For these data, $m = 4.6 \times 10^5$ and $q = -2.4 \times 10^6$. The numerical values for slope (m) and position (q) of the line are dependent on the unit of area chosen to express ED_{95}.

Equation (2) cannot be solved for G since the dose is included in both the reciprocal and logarithmic terms; however, the solution for r leads to

$$r = \left[\frac{3G}{4\pi d e^{1/mG - q/m}}\right]^{1/3} \qquad (3)$$

from which the curve in Figure 2 was obtained. The particle radius at which any given dose would provide 95 per cent disease control can be predicted by substituting the required values in (3).

In view of the properties of the regression equation and the errors inherent in the biological test methods, it is evident that a very large number of tests would have to be run before this relationship could be confirmed or disproved. The good agreement in this case must be to some degree fortuitous, since, even with the same data, discrepancies are evident at the ED_{50} level owing to unexplained variations in the slopes of the dosage-response curves. It becomes of interest, therefore, to examine the underlying assumptions that must be made to derive such a relationship, to determine whether it rests on a logical basis.

The regression equation (2) is approximately satisfied if two fundamental assumptions are accepted as describing the effects of particle size on the protection of foliage surfaces. These are: first, that the number of particles necessary to insure protection of each potential locus of infection is constant and independent of the dose, and, second, that on further subdivision of a constant weight of material, the rate of change in *apparent* dose with respect to the increase in the number of particles is inversely proportional to the number of particles present at the time of the change. These assumptions are intuitive, and to justify them it will be necessary to examine the premises on which they are based in some detail.

The first assumption is a simplification of a statistical problem. If an

area, S, representing the entire foliage surface, is divided into n sub-areas each of which is regarded as a potential locus of infection, then n particles would be required to protect the surface uniformly, providing that they could be arranged in an ordered pattern. Actually, the deposit is random, and follows a Poisson distribution. In the case of a small number of particles the chances are large that some sub-areas will be left unprotected, while others contain a superabundance of material, but if the number of particles is greatly increased the coverage will become more uniform. Ideal distributions can never be realized, and in fact are not required. As a first approximation, it is assumed that for any specific substrate-spore-toxicant combination, there exists a number of particles, N_o, which, when applied at random, will provide a deposit sufficiently uniform so that variations cannot be detected experimentally. This number, N_o, will be independent of the dose providing the particles are small compared to the distances between them, for if N_o small particles cover a surface satisfactorily, then N_o large particles should cover it equally well.

Consideration of the second assumption requires an explanation of the term *apparent dose*. A material applied to a foliage surface in the form of coarse particles would provide less effective disease control than if it were first ground to an optimum state of subdivision. It would, however, give control equivalent to a smaller amount of the optimum particle size preparation. Thus a deposit of 100 μg./cm.^{2} of coarse material might be equivalent in disease control to only 10 μg./cm.^{2} of the fine material. The actual dose, G, therefore would be 100 μg./cm.^{2}, while the apparent dose, A, would be 10 μg./cm.^{2} If the number of particles were increased through further subdivision of a constant amount of material, the apparent dose would increase until it equaled the actual dose as a limiting value. If G grams/cm.^{2} of a coarse material were further subdivided with the production of dN new particles, the apparent dose would increase by an amount dA owing to better distribution of the material and consequent protection of new loci against disease attack. However, the magnitude of the change in apparent dose would also be inversely proportional to the number of particles present at the time of the change. On an area which contained only a few large particles, the production of an additional one would materially improve coverage. However, if the number of particles were already large, and the surface close to saturation, the production of a single new particle at the expense of the entire population would have a relatively small effect. Thus the rate of change in apparent dose with respect to the number of particles is given by

$$\frac{dA}{dN} = \frac{k}{N} \qquad (4)$$

where k is a proportionality constant. When there is only one particle pres-

ent per unit of surface, the apparent dose will attain some very small value A_o depending on the size of the area chosen. Furthermore, as a first approximation we may let A_o equal the product of the weight of material G, and a very small constant ϵ. On integrating (4) between the limits ϵG and A, and $N = 1$ and N, we obtain

$$A = k \log_e N + \epsilon G. \tag{5}$$

The number of particles is given by equation (1), and substitution in (5) yields

$$A = k \log_e \frac{3G}{4\pi r^3 d} + \epsilon G. \tag{6}$$

When r attains some very small value r_o, the effective dose, A, will become equal to the actual dose, G, since the greatly increased number of particles will give optimum coverage. On applying this condition and solving for k, we obtain

$$k = \frac{G(1 - \epsilon)}{\log_e \dfrac{3G}{4\pi r_o^3 d}} \tag{7}$$

which on substitution in (6) yields

$$A = \left[\frac{G(1 - \epsilon)}{\log_e \dfrac{3G}{4\pi r_o^3 d}} \right] \log_e \frac{3G}{4\pi r^3 d} + \epsilon G. \tag{8}$$

At this point it is necessary to introduce the second of the above assumptions. If the number of particles required to attain optimum coverage is constant and independent of the dose, then for any dose a value for the radius will exist at which coverage will be perfect; or in general $log_e N_o = log_e 3G_n/4\pi r_n^3 d$ where N_o is a constant.

On applying this restriction to (8) and transposing the terms we obtain

$$\frac{1}{G} = \left[\frac{1 - \epsilon}{A \log_e N_o} \right] \log_e \frac{3G}{4\pi r^3 d} + \frac{\epsilon}{A} \tag{9}$$

for cases where $log_e N \leqq log_e N_o$. If the apparent dose A is held constant, equation (9) gives the relationship between particle radius and the dose required to maintain constant disease control, and on simplification of the constants is equivalent to the regression equation (2).

Thus, by making some simple primary assumptions it is possible to arrive at a relationship that will explain much of the experimental foliage data. The establishment of a satisfactory regression equation is not by it-

self important, for intermediate points in the dosage-response curves could be more easily determined by graphical interpolation. However, functions derived from it, and the principles upon which it is based, are useful in interpreting some of the data presented in the following sections.

CALCULATED AND OBSERVED PARTICLE SIZE EFFECTS

Since the amount of Phygon required to obtain 95 per cent disease control can be predicted for any particle radius by the use of equation (*2*), it becomes of interest to calculate the theoretical activity of a sample containing particles of many sizes, and to correlate the results with those obtained by tomato foliage tests. For the general case, it can be shown that where the particle size distribution curve is described by an analytic function $dp/dr = f(r)$ and the relationship of radius to amount of material required by $\Phi(r)$, the ED95 value for the dust is given by

$$L = \frac{1}{\int_{r_a}^{r_b} \frac{f(r)dr}{\Phi(r)}} \qquad (10)$$

where r_b and r_a are the upper and lower limits of the particle size distribution. Since the regression equation is implicit in G, and particle size distribution functions are usually complex, it is necessary to solve for L by finite increments, using the modified form

$$\frac{1}{L} = \sum_{r_a}^{r_b} \frac{\Delta P_i}{G_i}. \qquad (11)$$

In practice the distribution data are plotted in the form of a histogram in 1-micron intervals and the percentage of material in each range divided by the median value for G of that interval. The reciprocal of the sum of the terms for the entire distribution gives the calculated ED95 value for the dust.

It is usually not possible to make a direct comparison between calculated and observed ED95 values since the level of testing may vary owing to variations in response of the spores and plants in tests made at different times. However, in experiments which include a control sample, the relative effectiveness of a material in comparison to the standard is given by $R_o = ED95$ *of standard*$/ED95$ *of sample*. Correspondingly, a value for the calculated relative effectiveness, R_o, is given by the ratio of the values of L for the sample and standard determined from equation (*11*).

In order to test this relationship, a series of Phygon samples having widely different particle size distributions were prepared in a laboratory mill. Particle size measurements were made (6), and values for ED95 and relative effectiveness compared to a standard sample computed from the

TABLE III

CONTROL OF BLIGHT ON TOMATO FOLIAGE WITH GROUND PHYGON SAMPLES HAVING VARIOUS PARTICLE SIZE DISTRIBUTIONS

Av. radius, microns	Disease control (%) at concn., p.p.m.					ED_{95}, p.p.m.
	320	160	80	40	20	
2.3	100	100	100	93.6	84.7	37
3.5	100	100	99.1	95.9	78.8	42
3.7	99.9	99.8	94.7	83.6	72.1	60
5.2	100	99.8	84.5	76.4	50.5	79
9.5	100	97.8	85.6	67.9	67.9	100

histograms. Tomato plants were then sprayed in duplicate with each of the materials at concentrations of 240, 120, 60, 30, and 15 p.p.m. and control of *Alternaria solani* was determined by the usual methods. Leaf blight data are shown in Table III.

Subsequently, additional tests were run under equivalent conditions using these and other materials of known particle size distribution. Some additional materials had been run against the same standard at previous times, and these have been included in the compilation. Because of the

TABLE IV

COMPARISON OF CALCULATED AND OBSERVED EFFICIENCIES RELATIVE TO A STANDARD FOR PHYGON PREPARATIONS WITH VARIOUS PARTICLE SIZE DISTRIBUTIONS

Material	Test group	Efficiency ratios		Δ
		Calculated	Observed	
A	1	2.61	2.70	+0.09
A	2	2.61	2.50	−0.11
B	1	2.20	2.38	+0.18
C	1	1.93	1.67	−0.26
D	5	1.61	1.56	−0.05
E	1	1.39	1.27	−0.12
E	2	1.39	1.59	+0.20
F	3	1.25	0.93	−0.32
G	4	1.48	1.78	+0.30
H	2	1.44	1.39	−0.05
H	3	1.44	1.43	−0.01
H	4	1.44	1.39	−0.05
I	1	1.12	1.11	−0.01
J	4	0.96	0.78	−0.18

complexity of the distribution curves and the many steps necessary to convert all the particle size and foliage data to a common level, only the calculated and observed relative efficiencies are given in Table IV. Samples run at the same time are indicated by the test group numbers.

When calculated efficiencies were plotted against the observed values (Fig. 4), a straight line relationship was obtained with a moderate scattering of the points. The equation for the line determined by the least squares

method is $R_e = 1.06\ R_o - 0.126$. For ideal correlation, the slope should be unity and the position constant zero. While these data confirm the effect of particle size on biological activity, they do not necessarily demonstrate that the regression curve previously proposed to relate ED95 and radius is correct in detail. Presumably other relationships in which activity is an inverse function of radius would also lead to correlation, and it would be

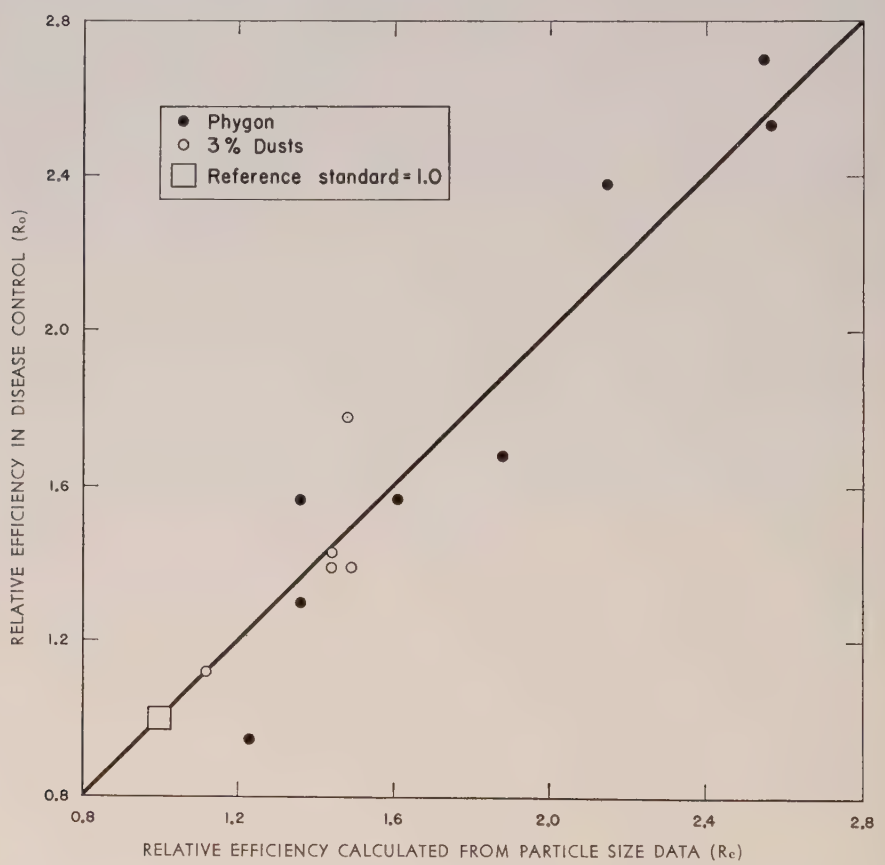


FIGURE 4. Correlation of experimental and calculated efficiencies of several formulations of dichloronaphthoquinone with different particle size distributions.

only by comparison of the correlation coefficients of the various possible curves that the best fit could be found. In the meantime, experimental errors and conditions beyond experimental control on the foliage surfaces would tend to overshadow these differences.

At the time these tests were carried out, it was assumed that variations in plant and spore viabliity were mainly responsible for the fact that

calculated ED95 values tended to be considerably higher than those obtained on foliage data.

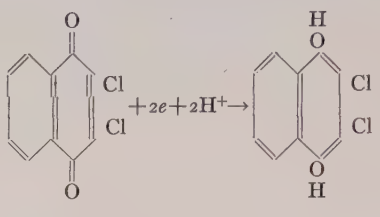
The blight control tests with the particle size fractions were run in a single group. The composite materials were tested subsequently at various intervals over a six-month period. During this time, infection became lighter and ED95 values for the standard sample varied by as much as 50 per cent. However, a re-examination of the data indicates an average difference of 65 per cent in calculated as compared to observed values. While the evidence is not conclusive, it appears that a synergistic effect might operate between particles of the various size classes to give better control than would be expected from the sum of the contributions of the components of the distribution. An effect such as this might be explained on the basis that the smaller particles serve to establish immediate control because of good coverage and greater availability at the sites of incipient infection, while the larger particles constitute a reserve supply of toxicant that will operate over a longer period owing to better resistance to erosion and photochemical deterioration. While the present data can do no more than suggest such a mechanism, it seems probable that the investigation of the fungitoxic properties of mixed size classes would be worth further work.

THE PROBLEM OF PERSISTENCE

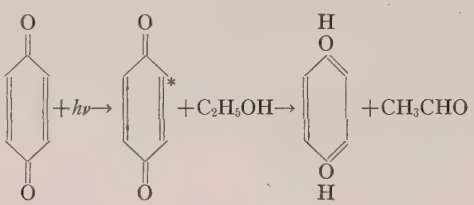
Tomato foliage tests carried out in the greenhouse focus attention only on the immediate effects of reduced particle size. Plants are inoculated as soon as the spray residues dry, and lesions are counted after a 4-day interval. In the field, conditions are much different. Here infection may occur at any time between spray applications or continuously throughout this interval. Furthermore, the deposits are exposed to unfiltered sunlight, so photochemical decomposition and interaction with constituents at the leaf surface may take place more rapidly. Chemicals with appreciable vapor pressure may be lost by sublimation when deposited in the form of very small particles while, conversely, finely divided materials may show better resistance to rainfall and wind. Ideally, the particle size effect should be expressed as an action integral describing the behavior of the fungicide over its useful life, rather than at some arbitrarily chosen time.

Quinones are known to be photochemically unstable. Leighton and Dresia (11) have shown that tetrachloro-*p*-benzoquinone in alcohol solution decomposes with a quantum efficiency of about 0.095. Experiments carried out in these laboratories using a polarographic method of analysis, show that dichloronaphthoquinone decomposes in acetone or dioxane solution when exposed to sunlight, but is comparatively stable in the dark. In one series of experiments, the quinone concentration of a solution of Phygon in dioxane was reduced to 6 per cent of its initial value after 17 days' exposure to diffuse sunlight.

The polarographic method of analysis (10) measures the current carried at a dropping mercury electrode during the conversion of a quinone to a hydroquinone.



The reaction is reversible, and a solution of the hydroquinone will give a well defined oxidation wave with the current flowing in the opposite direction. Ciamician and Silber (4) have found that in alcohol solution the main products from *p*-benzoquinone are acetaldehyde, hydroquinone, and a complex material, probably produced through reactions such as



In water, hydroquinone and a larger amount of the complex product are formed. Hartley and Leonard (8) found that the complex product has approximately the formula of a dimer.

Since the polarographic diffusion currents obtained on tetrachloro-*p*-benzoquinone and dichloronaphthoquinone are reduced to negligible values on prolonged exposure of their solutions to direct sunlight, it is evident that the quinone function is lost altogether since the formation of the corresponding hydroquinones would still give well defined waves.

Leighton and Dresia (11) have observed that the photochemical stability of the quinones is improved with increasing substitution. Thus, for the series *p*-benzoquinone, monochloro-, dichloro-, and tetrachloro-*p*-benzoquinones, quantum efficiencies decrease in the order 0.505, 0.354, 0.256, and 0.095. It has also been observed that the threshold wave length region required to obtain photochemical decomposition decreases with decreasing oxidation potential. Thus, tetrachloro-*p*-benzoquinone with an oxidation potential of 0.73 volt and a threshold region in the neighborhood of 5770 Å should be less stable to visible light than dichloronaphthoquinone with an oxidation potential of 0.42 volt.

Approximately 40 per cent of the total solar radiation is in the 4000–7600 Å region while 5 per cent or less is below 4000 Å (12, p. 69–71). Thus, a shift in the threshold region below this latter figure should promote better

field stability. Quantitative measurements of the decomposition rates of the two chemicals in dioxane solutions exposed to sunlight indicate that dichloronaphthoquinone is the more stable. This correlates well with field observations, for while tetrachloro-*p*-benzoquinone (Spergon) has found wide use as a seed protectant (19), it does not persist well on foliage. On the other hand, Phygon is a good seed protectant but has also found wide use as a spray material for fruit and field crops.

When a fungicide such as Phygon is distributed on fruit or foliage surfaces in the form of small discrete particles with very low solubility in the fluids in the environment, light stability would be expected to be very much better than in solution. Only the soluble material and surface layers would be expected to react, while the interiors of the particles would maintain a reservoir of unaltered material capable of renewing the fungicidal action. When particle size is drastically reduced, the specific surface and consequently the rate of solution would become much greater. While reducing particle size improves coverage and immediate disease control, if carried to extremes it might result in deposits with poor persistence. It is necessary, therefore, to balance these two factors in order to obtain a formulation with optimum properties.

The total surface of a fungicide in a spray deposit at 95 per cent disease control in terms of square centimeters per square centimeter leaf area is given by

$$S = \frac{3G}{rd} \tag{12}$$

where S is the surface, G the amount of material, r the particle radius, and d the density of the material.

On substituting (12) in the regression equation (2) and solving for S, we obtain an expression relating total surface to radius and dosage at 95 per cent disease control.

$$S = 4\pi r^2 e^{1/mG - q/m}. \tag{13}$$

On substituting values for r and G in equation (12) or (13) and plotting total surface against radius (Fig. 5) a U-shaped curve is obtained with a minimum in the 2 to 6 micron region.

The exact value of the minimum can be found in the following way. On substituting (3) in (13) the radius term is eliminated and the surface expressed as a function of dosage:

$$S = \frac{4\pi [3G]^{2/3} e^{1/mG - q/m}}{[4\pi d]^{2/3} [e^{1/mG - q/m}]}. \tag{14}$$

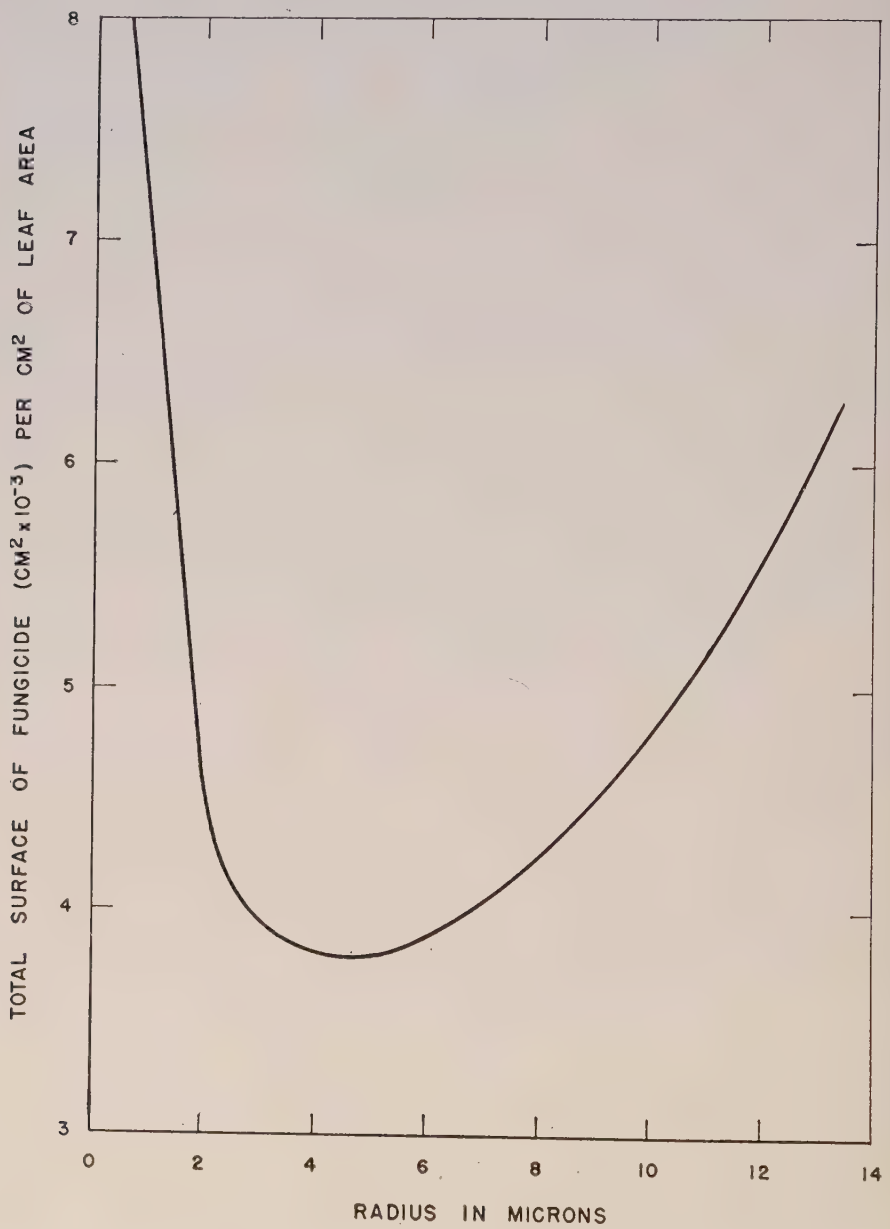


FIGURE 5. Relationship between total surface and size of particles at 95 per cent disease control (equation *12*).

On differentiating (*14*) with respect to G, the derivative is

$$\frac{dS}{dG} = \frac{2K\left[e^{1/mG - q/m}\right]^{1/3}}{3G^{1/3}} - \frac{KG^{2/3}e^{1/mG - q/m}}{3mG^2} \qquad (15)$$

$$\text{where } K = \frac{4\pi 3^{2/3}}{(4\pi d)^{2/3}}.$$

On permitting $ds/dG = 0$, where the total surface is a minimum, we obtain

$$G = \frac{1}{2m}. \qquad (16)$$

On substituting (*16*) in (*3*) the value for the radius at which 95 per cent disease control is attained with minimum total surface is

$$r = \left[\frac{3}{8\pi mde^{2-q/m}}\right]^{1/3} = 4.9\mu. \qquad (17)$$

The curve in Figure 5 requires some explanation. When the particle size is large, the specific surface is small. However, so much material is required to give 95 per cent disease control that the total surface of the deposit per unit leaf area is relatively large. As the particle size is reduced the coverage factor increases faster than the specific surface, and total surface required for 95 per cent control decreases. Finally when the coverage factor begins to approach its limiting value, the effect of greatly increased specific surface predominates, and the total surface again increases.

It is somewhere in the minimum range of the curve that the best combination of effective dose and total surface should be found, for at the larger radii the material is not used efficiently, and at the lower values a disproportionately large area of chemical is exposed to deteriorating influences for only moderate gains in coverage.

This supposition is partly confirmed by some tomato foliage tests carried out at the same time as the initial work on particle size effects. Each particle size fraction was sprayed at concentrations of 400, 200, 100, 50, 25, 12.5, and 6.25 p.p.m. on triplicate plants and held under greenhouse conditions for one week before inoculation with spores of *Alternaria solani*. During this interval, a loss in disease control was noted, presumably due to deterioration of the chemical and to some extent to increase in leaflet area during growth. The ED95 values for the weathered plants (Table V) were calculated on the assumption that the doses were equivalent to those in the unweathered series, and the material lost in effectiveness. Actually, the remaining material was probably still equally effective,

but there was less of it present at the sites of infection owing to leaflet growth and decomposition to non-fungitoxic materials.

The percentage losses indicate that material in the 1.5 to 3.5μ radius range was the most stable. After weathering, the 1.5μ particles gave the best disease control. The control on samples less than 1μ was considerably reduced on a percentage basis, but the absolute values are still satisfactory. It should be remembered, however, that the tests were run in a greenhouse during the winter months where photochemical effects would be much less serious than in the field during the growing season. The reasons for the

TABLE V

LOSS IN EFFECTIVENESS OF PARTICLE SIZE FRACTIONS OF PHYGON AFTER GREENHOUSE WEATHERING OF SPRAYED TOMATO PLANTS

Particle size, microns	ED95, p.p.m.		Per cent of activity lost
	Initial	After weathering	
0.45	52	240	79
0.81	64	300	79
1.48	89	135	33
3.57	157	220	28
8.36	445	—	100
13.8	870	—	100
24.5	2,200	—	100

complete loss in activity of the large particle size fractions are not entirely clear since the eroded surfaces would be expected to be renewable over a long period owing to their relatively large volumes. It may be that tenacity to the foliage surfaces was poor, and they were dislodged during the growth of the leaflets, or that coverage was so poor, deterioration coupled with an increase in leaf area gave a complete loss of control.

The range for the best over-all utilization appears to be somewhat lower than the value of 4.9μ predicted from equation (*17*). This value is at best approximate and must be considered with reference to the photochemical stability of the material. However, it seems likely that maximum efficiency of utilization should be obtained somewhere in this range.

PARTICLE SIZE IN SEED PROTECTANTS

In the derivation of equation (*9*) relating ED95 to particle radius, two assumptions were made regarding the mode of action of the fungicide, the first of which is directly related to problems in seed treatment. It was stated that the number of particles required to insure protection of each potential locus of infection is constant and independent of the dose.

It is understood that the nature of the surface and the organism causing infection remain unchanged, and that the sizes of the particles are small

relative to the distances between them. This amounts to saying that for a spore capable of invading a fixed sub-area, there exists a state of subdivision for the fungicide so that when it is applied at random to the total area, the chance that some sub-area will be left unprotected is vanishingly small. If the particles themselves are extremely small, effective disease control might not be maintained, but this would be the fault of insufficient dosage rather than imperfect coverage. Presumably perfect coverage could be obtained with a fixed number of mathematical points or with a non-fungicidal material. There would be no disease control but conditions for the saturation of the surface at possible infection loci would be fulfilled. As the sizes of the particles became large relative to the distances between them, deviations in this value would be expected since the particles themselves would cover a fairly large area and fewer of them would be required to saturate the surface.

An important corollary of this proposition is that there is more to be gained from reducing particle size of a highly potent fungicide than of a weak one. A material with very low toxicity would have to be applied at such high doses (to obtain any disease control at all) that the surface would be saturated with respect to coverage at comparatively large particle sizes, and there would be little point to further subdivision. Conversely, as the innate toxicity of the chemical increases, greater care must be taken to disperse the smaller amount of material so that most of the infection loci are protected.

Foliage data are not available comparing the effects of particle size reduction on materials of widely differing innate toxicity. However, a comparison of the foliage and seed protective abilities of Phygon offers some points in similarity. Phygon is ordinarily used on seed at a dosage of 0.12 per cent by weight. A 100 g. lot of pea seed would have a total surface area in the neighborhood of 700 cm.^{2} At a particle radius of 10μ there would then be 2.5×10^4 particles of Phygon per square centimeter of seed surface. This is far in excess of the number that would be deposited on foliage at ED95, and would be expected to saturate the surface to an extent that further reductions in particle size would have little effect.

In order to verify this supposition, several of the Phygon dusts previously evaluated on foliage were applied to pea seed at doses of 0.25, 0.12, 0.06, 0.03, and 0.01 per cent by weight. The data on emergence in Table VI show no significant difference between the samples with different particle sizes. Subsequently a series of fractions of tetrachloro-*p*-benzoquinone (Spergon) covering the range from 1 to 35μ in small intervals, were prepared by fractional sedimentation and tested on pea seed. Results on the fractions with very large mean radii showed some lack in control, but the effect was much less pronounced than on foliage and may have been caused in part by poor adhesion of the larger particles to the seed surface. Calcu-

lated values for the number of particles indicate that the surfaces should be well protected in all cases. With some materials, very small particle sizes might have a deleterious effect owing to their rapid erosion in the soil. In the formulation of seed protectants, the principal consideration should be to obtain a particle size that will give good adhesion to the seed, satisfactory flowability in treating equipment, and good stability in the soil, with only secondary emphasis on coverage factors.

SOME DERIVED FUNCTIONS

One of the advantages of a relationship between particle radius and dosage is to supply a means for the examination of situations where experimental evidence is unattainable, or would require an impractically large

TABLE VI

EFFECT OF PARTICLE SIZE OF PHYGON ON EMERGENCE OF PEAS

Av. radius, microns	Per cent emergence at various dosages*				
	0.25	0.12	0.06	0.03	0.01
2.3	90	90	88	65	60
3.7	93	84	89	76	73
5.2	86	89	85	83	66
9.5	90	81	79	81	70

* Expressed as per cent by weight.

number of tests. The conclusions derived therefrom would be only as reliable as the initial data and must be viewed in that light.

A general statement of the particle size effect requires an equation in three variables: disease control, dosage, and radius. The former two quantities are related by an empirical expression proposed by Bliss (1) which was later adopted for the interpretation of leaf blight data (15).

$$p = a + b \log_{10} G. \qquad (18)$$

In this equation, p is the disease control expressed in terms of probit units, or units of standard deviation $+5$, G is the dose, a is a constant describing the level of toxicity, and b is the slope of the regression curve which describes the rate of change of disease control with respect to dosage. The use of probit units to express disease control is based on the observation that individuals in a spore population show variations in their resistance to toxic agents, and that this variation assumes the form of a probability distribution curve. Thus, a larger number of individuals are affected by a change in dosage in the region of ED50 than by a corresponding change at ED10 or ED95. The reasons for the logarithmic dependency of the dosage are less firmly established; however, according to Bliss (1) an answer "may

be sought in the relation between the dosage administered and the amount of poison fixed by the essential cells or tissues." Although this equation cannot be justified on theoretical grounds, it has had wide acceptance in the interpretation of dosage-mortality data.

Since there should be no difference between the operation of an actual dose and an apparent dose, it appears likely that the probit disease control should be related to the effective dose A in (9) by an equation of the type:

$$p = \alpha + \beta \log_{10}\left[\frac{G(1-\epsilon)}{\log_{10} N_o}\log_{10}\frac{3G}{4\pi r^3 d} + \epsilon\right]. \qquad (19)$$

On combining the constants and setting ϵ equal to zero, equation (19) assumes the form

$$p = \Psi + \beta \log_{10}\left[G\log_{10}\frac{3G}{4\pi r^3 d}\right]. \qquad (20)$$

Equation (20) implies that the number of new particles as well as the increase in over-all dosage should be considered in the interpretation of dosage-response data. This conception is at variance with ordinary usage, but may have some basis in fact. If the total weight of material were increased while keeping the number of particles constant, coverage, and consequently disease control, would not improve so rapidly as if the material were added in the form of new particles. In the latter case, each additional amount of material would protect new loci against infection as well as contribute to the total weight of toxicant on the foliage surface.

In regions of intermediate particle size, both variations in spore resistance and variations in coverage will affect disease control. As dosage is increased at constant particle size, the more resistant spores will be inactivated, and at the same time more of the possible areas of infection will be protected.

Calculated functions for the two forms of the dosage-response curve for the 0.45μ Phygon particle size fraction are shown in Table VII. On

TABLE VII

DERIVED DOSAGE-RESPONSE UNITS FOR 0.45μ PHYGON PARTICLE SIZE FRACTION

Deposit μg./cm.^{2}	% Disease control	Probit	$N \times 10^{-5}$	$\log_{10} G$	$\log_{10}(G \log_{10} N)$
2.16	100.00	—	34.3	−5.67	−4.85
1.08	99.97	8.43	17.2	−5.97	−5.17
.54	99.10	7.37	8.59	−6.27	−5.50
.27	93.20	6.49	4.29	−6.57	−5.82
.14	85.10	6.04	2.15	−6.87	−6.14
.068	19.20	4.13	1.07	−7.17	−6.47

plotting these data, satisfactory linear relationships are obtained in both cases (Fig. 6). It is apparent that the interrelationship between disease control, dosage, and coverage can be represented to a reasonably close

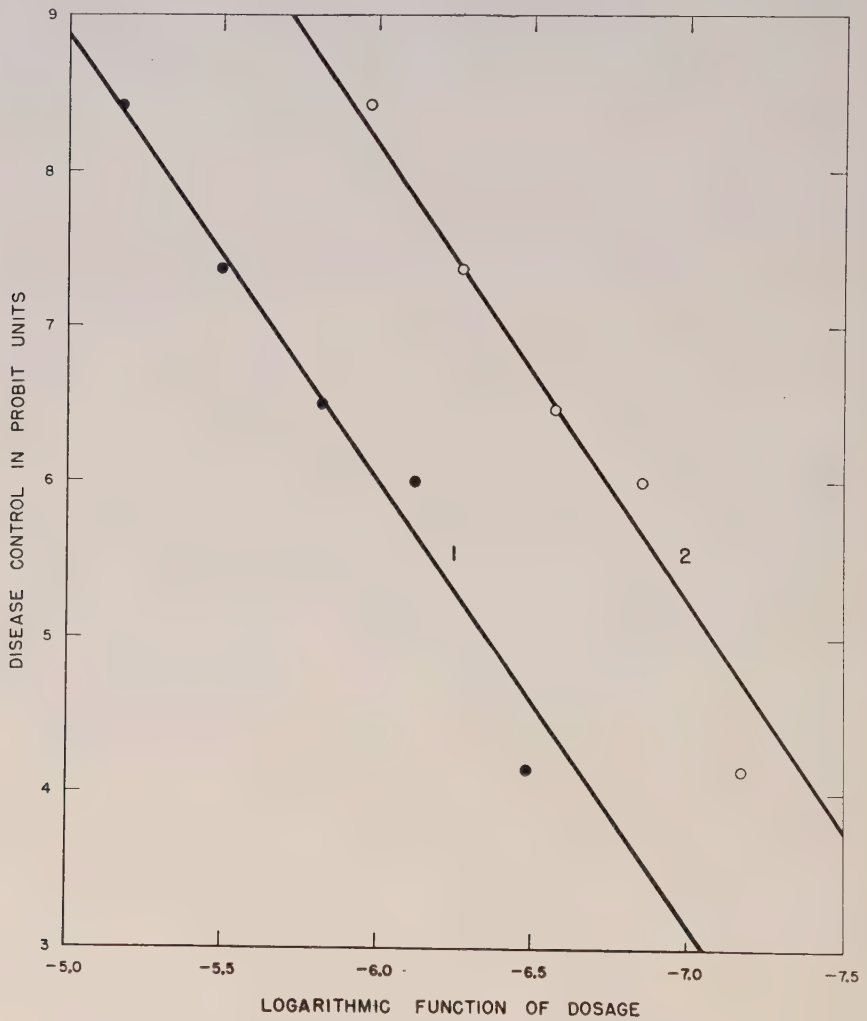


FIGURE 6. Dosage-response curves for 0.45μ Phygon. 1, $log_{10}(G \log N)$ vs. control (equation 20). 2, $log_{10}G$ vs. control (equation 18).

approximation by this method. A complete interpretation of the data in this form would require some adjustments in units of measurement since low doses in the higher particle size regions would, in some cases, contain less than one particle per square centimeter.

In addition to the foregoing relationship, it is also possible to obtain a

physical picture of certain other properties through the use of equation (*20*). Thus, if the dose is held constant and the radius decreased, the improvement in disease control with the number of particles per unit area can be estimated (Fig. 7). By an extension of this treatment, it is possible to obtain information on the regression of probit control with surface (Fig. 8). While these and many other properties of dispersed fungicides can be interpreted in this manner, it must be kept in mind that the pictures are only valid insofar as the underlying postulates can be justified.

PRECISION OF MEASUREMENTS

The ED_{95} values expressed as parts per million in the spray suspensions were calculated according to the probit method of Bliss (1). Six concentrations were run on each sample, and each concentration replicated with four plants. Data at each point were weighted according to the empirical method described by McCallan (14). Each ED_{95} value is thus the

TABLE VIII

VARIANCE IN ED_{95} VALUES AND SLOPES OF DOSAGE-RESPONSE CURVES

Mean radius, microns	ED_{95}, p.p.m.	ED_{95}, range	Slope	Variance in slope	χ^2
0.45	52	46– 57	3.0	0.29	7.2
0.81	64	60– 67	2.5	0.04	1.9
1.48	89	60– 130	2.5	2.44	147.6
3.57	157	110– 175	1.7	0.05	5.8
8.39	445	375– 520	3.0	0.48	7.8
13.8	870	715–1,050	3.5	0.57	4.8
24.5	2,200	—	2.0	—	—

result of 24 separate observations. χ^2 values for the different materials (Table VIII) are reasonably uniform except for the 1.48μ fraction. The ED_{95} value for the 24.5μ fraction was estimated since the points were scattered, and well below the range for good disease control.

Although Bliss' method of calculation is generally accepted, it should be pointed out that it is not strictly applicable when doses are applied by the spray method, for it is assumed that the doses are accurately known when, in fact, they are not. The coefficient of deposition obtained by analytical methods was found to be 5.40×10^{-3} μg./cm.^{2} with a standard deviation of 0.51×10^{-3}. Thus, under carefully controlled conditions a 10 per cent error in dosage is not uncommon, and larger errors may be encountered under routine testing procedures. Data in Table I also indicate that leaf areas vary considerably from plant to plant. Since the number of lesions observed would be expected to increase with larger leaf surfaces, this may be a considerable source of error in the calculation of disease control.

In fitting the data to the regression equation (*2*), another situation

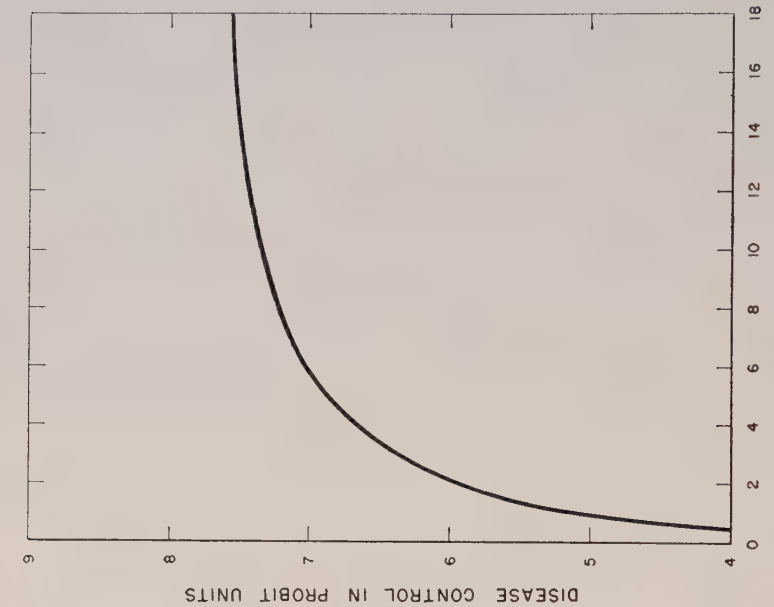


FIGURE 8. Relationship between probit control and total surface of the fungicide at a concentration corresponding to 100 parts per million in the spray suspension.

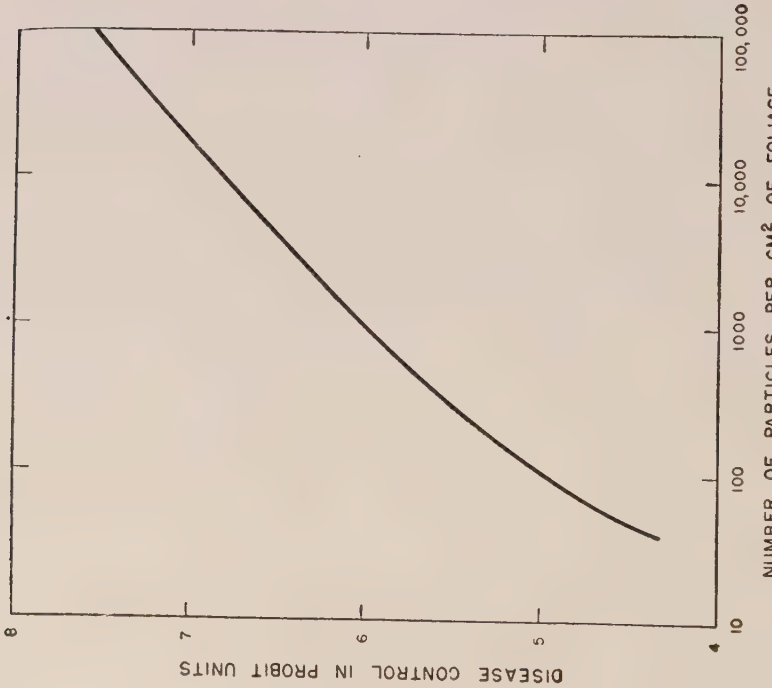


FIGURE 7. Relationship between number of particles and probit disease control at a spray suspension concentration of 100 parts per million.

arises in which errors are present in both variables. The error in the reciprocal of the dosage is given by

$$\Delta\left(\frac{1}{G}\right) = \frac{\Delta G}{G^2} \tag{21}$$

and the error in the number of particles by

$$\Delta(\log_e N) = \left[\left(\frac{\Delta G}{G}\right)^2 + \left(\frac{3\Delta r}{r}\right)^2\right]^{1/2}. \tag{22}$$

The points in Figure 3 represent the experimental values, and the rectangles, the zones of error calculated from equations (*21*) and (*22*).

Observed and calculated values for ED95 and ED50 are shown in Table IX. The ED50 values were calculated by assuming an average slope of 2.5 for the Phygon dosage-response curve. The weighted average for the

TABLE IX

CALCULATED AND EXPERIMENTAL VALUES FOR BLIGHT CONTROL WITH PHYGON PARTICLE SIZE FRACTIONS

Radius, microns	ED95, μg./cm.²		ED50, μg./cm.²	
	Exptl.	Calcd.	Exptl.	Calcd.
0.45	0.28	0.28	0.08	0.06
0.81	0.35	0.34	0.08	0.07
1.48	0.48	0.47	0.11	0.10
3.57	0.85	0.83	0.10	0.17
8.36	2.40	2.14	0.65	0.47
13.4	4.70	5.25	1.62	1.20
24.5	12	23	3.16	5.08

experimental slopes is in the neighborhood of 2. However, this is contributed to by the very flat slope and good fit of the 3.57μ fraction. Since this curve is known to be atypical, it was rejected despite the good precision of measurements.

Calculated and observed results at the ED95 level agree very well except at the larger particle sizes. However, values on samples above 10μ were obtained by extrapolation, and it also must be considered that an equation based on differentials will give only approximate results when applied to situations where there are only a small number of particles. Results at the ED50 level are less satisfactory although they show some correspondence when large changes in radius are considered. The ED50 measurements are in a region of lower statistical significance on tomato foliage tests (14) and reflect to a greater extent unexplainable variations in the slopes of the dosage-response curves.

DISCUSSION

The chemical processes by which the fungicide inactivates the spores have not been taken into account and they are not relevant to this discussion except for a consideration of transfer mechanisms. The foliage surface can be visualized as a two dimensional system in which particles of an insoluble fungicide are distributed at random to protect against invasion from fungus spores, which are also distributed at random. There are several mechanisms by which this process might operate, and there is no assurance that the action is always the same with different materials. Probably, direct contact between particles and spores is not required for all fungicides, since scale drawings of Phygon (Fig. 1) indicate this would be very unlikely on foliage protected by large particles.

There is, however, the possibility that a spore will germinate and send out hyphae that encounter particles of toxicant before they can penetrate the leaf tissue. If the particles are large enough, or if the hyphae reach a great many small particles, the entire organism might be inactivated before infection. The fungicide could also operate by slow solubilization and diffusion into adjoining areas of the surface. A large particle would operate more effectively over a limited range than a small one, but the average locus of infection would not be so well protected since the concentration of solubilized toxicant would drop off rapidly as the distance from the source increased owing to diffusion, discontinuities in the surface, or decomposition of the soluble phase. McCallan (13) and McCallan and Wilcoxon (16) have proposed that fungus secretions diffuse into the immediate vicinity of the toxicant where they react chemically to form intermediate compounds that inactivate the spores when they are reabsorbed. More steps would be involved in this process; however, the fundamental distribution problem remains the same. Therapeutic action through translocation of the chemical or a reaction product through the plant system has also been considered. General systemic protection seems unlikely, since good distribution must be maintained to prevent infection. If this type of action does occur, it must be effective only at short distances from the individual particles, beyond which the chemical is dissipated by diffusion and reaction with materials in the plant cells. All of the mechanisms proposed appear to require good distribution for adequate protection.

It is clear that the distribution effect must be expressed by a function that reaches a limiting value. If disease control were directly dependent on surface, number of particles, or radius, the toxic effect could be increased indefinitely by subdivision down to molecular size. The innate toxicity of a chemical is not changed by fragmentation, for a given weight of material is capable of inactivating only a limited number of spores. By increasing the state of subdivision, it is possible to obtain more effective utilization,

but this must soon reach a limiting value as the surface approaches saturation. The region in which this will occur probably varies with the characteristics of the spore, foliage type, chemical, and method of application. The primary consideration is the size of the unprotected area that must be available for a successful invasion by the fungus. If the variation in protection in each sub-area is small compared to the average dosage per subarea, then the fungicide will be used effectively. In a region of very small particle size where the coverage is already good, gains in control through further subdivision will be small. The system has reached a point of diminishing returns which is most easily expressed by a logarithmic relationship.

The number of particles per unit area rather than total surface has been regarded as the primary factor in disease control. Increasing the surface will increase the rate of solubilization of toxicant and so, in a sense, increase availability. In a continuous three dimensional system, agitated to prevent local concentration effects, the rate of solution of a particulate solid is directly related to the surface as well as the concentration of solute in the aqueous phase. In glass slide tests, these conditions are more nearly fulfilled than on foliage. Even here the positions of the particles are fixed, and the diffusion of the solute to a homogenous equilibrium concentration must be slow. Microscopic examination of test slides has shown that spores in the immediate vicinity of a large Phygon particle are inhibited, while those some distance away will germinate. It is evident that proximity of a particle of the solid fungicide is necessary for fungistasis even in this case. On the essentially two dimensional surface of a leaflet, the particles are in fixed positions and relatively far apart. Diffusion of toxicant from one location to another will be a slow process interrupted by discontinuities on the surface and decomposition of the soluble phase in the presence of light and plant excretions. The only way by which the entire area open to infection can be protected is by fragmentation and random distribution of the toxicant, and this is primarily a function of the number of particles available rather than total surface of the fungicide.

A small number of large particles will lead to a superabundance of protectant in some localities, and little or none in others, while subdividing the material will tend to equalize the coverage at the various points of infection. If the total dose is very low, and the deposit is uniform, only that percentage of the spore population with high susceptibility to the toxicant will be inactivated. Increasing particle size may prevent germination of the more viable spores at a few scattered points, but this will be more than overbalanced by a total absence of protectant at other loci. It might be argued that for extremely minute dosages, large particles would be more effective, since there would be a finite chance that one of them would be present at an infection locus in sufficient quantity to inactivate

the spore. However, the variance in the resistance of the spore population is represented by a probability distribution which implies that some spores will be affected at the smallest average doses.

If sufficient data were available on the range and intensity of action of a single fungicide particle with respect to its size, and the concentration of soluble material over a time interval necessary to inactivate a segment of the spore population, a more complete statement of the coverage problem might be made through statistical methods. However, this would entail consideration of many unresolved factors, and involve probability distributions for both the variance in surface coverage, and the variance in the spore population.

In considering the effects of coverage, the fungicide particles are assumed to be distributed at random on the foliage surface. In practice, they probably are not, which might be one of the factors leading to experimental discrepancies. Spray suspensions are prepared with sufficient surface-active agent to prevent agglomeration. However, the material is delivered from a spray gun and deposited on the foliage as fine droplets. On drying, these droplets are likely to leave non-uniform residues. If this is the case, the particle size effect would be partly masked, for coverage would not be completely satisfactory even with the optimum number of particles. Lesions which appear on some plants could be caused by irregularities in the deposit rather than insufficient dosage or particle size effects. Applying the fungicide as a dust should, in theory, give a more nearly random distribution. However, it would be difficult to insure a constant deposit with the various size classes owing to different sedimentation rates. In spray tests carried out under uniform conditions, the chances are good that irregularities in deposit will occur in similar patterns for the various size classes. Although disease control might be lower than expected from random coverage, the errors would all be in the same direction, and the relative values approximately correct.

A general relationship has been proposed which states that the amount of toxicant required for constant disease control varies inversely with the logarithm of the number of particles into which it is subdivided. Experiments made on tomato foliage tend to substantiate this at the ED_{95} level although there are discrepancies at lower values owing to variations in the slopes of the dosage-response curves. The regression equation gives a static rather than a dynamic picture of the situation since it does not take into account the exhaustion of the smaller particles with time owing to photochemical deterioration and other possible erosion effects. In greenhouse foliage tests, spray and inoculation schedules are arranged to give data on the immediate protective action of the fungicide. However, even here, considerable time elapses between spraying and counting of the lesions, during which secondary effects may become apparent.

The relationship between particle size and dosage is useful for the correlation of test results and the interpolation of intermediate points in the data. However, it has more value as a starting point for speculations on the mechanism of fungicidal action, and the postulates on which it is based can serve as useful guides for the formulation of new and existing materials. Weak fungicides which can be used in quantity because of low cost and absence of phytotoxic effects are not likely to be improved quite so much by extreme subdivision as materials with very high innate toxicity. On greenhouse tomato foliage tests, Phygon gives satisfactory coverage with 1.5μ particles at a dose of 89 p.p.m. As a first approximation, a fungicide with one-fifth its innate toxicity would give equivalent coverage at 2.6μ, and a material with one-tenth the toxicity at 3.2μ. Conversely, a material ten times as toxic as Phygon would have to be ground to a 0.7μ dust to insure efficient distribution. In the treatment of seed, where the dosage is of a higher order of magnitude, particle size effects are relatively unimportant. These estimates do not take into account possible differences in the modes of assimilation by the fungus spores where coverage requirements might be different, nor are they intended to translate laboratory data to field performance.

Pronounced differences in the solubility of chemicals might suppress particle size effects to a great extent. It has been observed that some of the derivatives of 4-nitrosopyrazole (18) are moderately soluble in water. When spray suspensions are prepared by throwing the compounds out of acetone solution by the addition of water, dispersion tends to be poor because of crystal growth. Yet these compounds give very low ED95 values on tomato foliage. In cases such as these, rapid solubilization and diffusion at the foliage surface might tend to overcome some of the effects of poor particle size distribution. However, in general, liquids, and highly soluble solids, have not been successful as commercial fungicides.

The performance of a dust containing particles of all sizes cannot be predicted from knowledge of a single characteristic such as average radius. The observed effect is a summation of all the contributions made by the individual size classes, and their magnitudes do not vary linearly with radius. Furthermore, any number of particle size distributions can exist for a single value for average radius, and the shapes of these curves will determine the net over-all effects. There is some evidence of complementary action between particles of various size classes; nevertheless an adequate estimate of relative performance can be obtained from the distribution curve of a dust.

In making particle size evaluations, it is necessary to consider the chemical properties of the fungicide in relation to the degree of subdivision. Rate of utilization increases very rapidly with decreasing radius down to a region somewhere in the vicinity of 5μ. After this, the rate falls off. Further

reduction in size will greatly increase surface without commensurate gains in efficiency. If the material is volatile, or inherently unstable, in the environment at the foliage surface, persistence might be sacrificed for immediate gains in control.

SUMMARY

1. The amount of Phygon (2,3-dichloro-1,4-naphthoquinone) required to control tomato early blight is inversely proportional to the logarithm of the number of particles into which it is subdivided. This equation can be derived on a theoretical basis providing several assumptions are made regarding the mode of action of particulate fungicides.

2. The performance of a dust relative to a standard can be predicted from its particle size distribution curve and the regression of ED95 with particle radius. Calculated and observed results agree to within the limitations of the experimental methods.

3. A curve relating particle radius and total surface of a fungicide at ED95 is derived which exhibits a minimum near 5μ. It is believed that photochemically sensitive materials can be used most effectively in this range since a low ED95 is obtained in combination with minimum exposed surface.

4. Reducing the particle size of Phygon is not as effective in preventing seed decay as it is on leaf blight control. This may be related to the comparatively large doses required in seed treatment with consequent saturation of the surface at large particle size.

5. The regression of ED95 with particle radius is useful for interpreting interrelationships between dosage, response, specific surface, and number of particles which are often not accessible to direct experimentation.

6. Various mechanisms which can be used to explain the particle size effect are discussed in detail.

7. A consideration of particle size effects together with information on the chemical and physical properties of the toxicant may often be useful in preparing new fungicidal formulations.

LITERATURE CITED

1. BLISS, C. I. The calculation of the dosage-mortality curve. Ann. App. Biol. **22**: 134–167. 1935.
2. BURCHFIELD, H. P., DELORA K. GULLSTROM, and G. L. MCNEW. Agricultural dusts. Preparation of dusts of uniform particle size by fractional sedimentation. Analyt. Chem. **20**: 1168–1173. 1948.
3. BURCHFIELD, H. P., and G. L. MCNEW. The colorimetric determination of 2,3-dichloro-1,4-naphthoquinone on seed. Phytopath. **38**: 665–669. 1948.
4. CIAMICIAN, GIACOMO, und P. SILBER. Chemische Lichtwirkungen. Ber. Deutsch. Chem. Ges. **34**: 1530–1543. 1901.
5. FEICHTMEIR, EDMUND F. The effect of particle size and solubility of sulfur in carbon disulfide upon its toxicity to fungi. Phytopath. **39**: 605–615. 1949.

6. GULLSTROM, DELORA K., and H. P. BURCHFIELD. Agricultural dusts. Determination of particle size distribution. Analyt. Chem. **20**: 1174–1177. 1948.
7. HAMILTON, J. M., D. H. PALMITER, and G. L. MACK. Particle size of sulphur and copper fungicides in relation to apple scab and cedar-apple rust control. Phytopath. **33**: 533–550. 1943.
8. HARTLEY, WALTER NOEL, and ALFRED GODFREY GORDON LEONARD. The absorption spectra of *para*-benzoquinone, quinol, and quinhydrone in the state of vapour and in solution. Jour Chem. Soc. **95**: 34–52. 1909.
9. HEUBERGER, JOHN W., and JAMES G. HORSFALL. Relation of particle size and color to fungicidal and protective value of cuprous oxides. Phytopath. **29**: 303–321. 1939.
10. KOLTHOFF, I. M., and J. J. LINGANE. Polarography. 510 pp. Interscience Publishers Inc., New York. 1941.
11. LEIGHTON, PHILIP A., and WILLIAM F. DRESIA. The effects of substituents on quantum efficiency in the quinone-alcohol reaction. Jour. Amer. Chem. Soc. **52**: 3556–3562. 1930.
12. LUCKIESH, M. Artificial sunlight. 254 pp. D. Van Nostrand Co., New York. 1930.
13. MCCALLAN, S. E. A. Studies on fungicides. III. The solvent action of spore excretions and other agencies on protective copper fungicides. *In* New York [Cornell] Agric. Exp. Sta. Mem. 128: 25–79. 1930.
14. ————. Empirical probit weights for dosage-response curves of greenhouse tomato foliage diseases. Contrib. Boyce Thompson Inst. **13**: 177–183. 1943.
15. MCCALLAN, S. E. A., and R. H. WELLMAN. A greenhouse method of evaluating fungicides by means of tomato foliage diseases. Contrib. Boyce Thompson Inst. **13**: 93–134. 1943.
16. MCCALLAN, S. E. A., and FRANK WILCOXON. The action of fungous spores on Bordeaux mixture. Contrib. Boyce Thompson Inst. **8**: 151–165. 1936.
17. MCNEW, GEORGE L., and H. P. BURCHFIELD. Particle size in relation to the fungitoxicity of dichloronaphthoquinone. Contrib. Boyce Thompson Inst. **16**: 163–176. 1950.
18. MCNEW, GEORGE L., and NORMAN K. SUNDHOLM. The fungicidal activity of substituted pyrazoles and related compounds. Phytopath. **39**: 721–751. 1949.
19. SCHOENE, D. L., H. DOUGLAS TATE, and T. W. BRASFIELD. Use of quinones as fungicides. Agric. Chem. **4**(11): 24–27, 73, 75–77. 1949.
20. TWENTYMAN, R. L. Experiments on the control of "stinking" smut or bunt. Part 2.Tests of the dry copper powders. Jour. Dept. Agric. Victoria **29**: 235–248. 1931.
21. WILCOXON, FRANK, and S. E. A. MCCALLAN. The fungicidal action of sulphur. III. Physical factors affecting the efficiency of dusts. Contrib. Boyce Thompson Inst. **3**: 509–528. 1931.

PARTICLE SIZE IN RELATION TO THE FUNGITOXICITY OF DICHLORONAPHTHOQUINONE

GEORGE L. MCNEW AND H. P. BURCHFIELD[1]

Investigations were begun in 1946 to develop an all-purpose dust that could be used to control both disease and insect pests of garden plants. After studying various mixtures, 2,3-dichloro-1,4-naphthoquinone[2] was chosen as the fungitoxicant and rotenone as the insecticide. Several formulations of these materials, with and without sulfur and DDT supplements, were blended with talc in such proportions that they could be applied as dusts at 40 to 60 lb. per acre or as sprays at a concentration of about 20 lb. in 100 gallons of water.

In field trials, these compositions proved to be about twice as effective per pound of fungitoxicant as wettable dichloronaphthoquinone (Phygon) in preventing the late blight of potatoes and tomatoes caused by *Phytophthora infestans* (Mont.) DeB. Since particles of the fungitoxicant had ample opportunity to fragment during blending operations, it was assumed that this unexpected superiority might have been caused by decreased particle size rather than other effects such as synergism, greater adherence or more uniform distribution on leaf and fruit surfaces.

Studies were undertaken, therefore, to determine whether particle size distribution differed appreciably from that in the parent material and, if so, how much effect particle size had upon fungitoxicity. The results of field, laboratory, and greenhouse trials with different formulations and fractions of dichloronaphthoquinone are presented in this paper.

MATERIALS AND METHODS

Three different dust-spray formulations of dichloronaphthoquinone were tested alongside the relatively pure chemical (wettable Phygon) and Bordeaux mixture (4-2-50) on potatoes (*Solanum tuberosum* L.) of the variety Green Mountain and tomatoes (*Lycopersicon esculentum* Mill.) of the variety John Baer at Bethany, Conn., in 1946. The formulations of these three dusts are given in Table I.

The dust-sprays were tested as spray materials at dosages of 30, 20, and 10 lb. per 100 gallons of water (equivalent to 0.9, 0.6, and 0.3 lb. of dichloronaphthoquinone). All sprays were applied at a rate of 150 to 200 gallons per acre by conventional hydraulic equipment operated at 350 lb.

[1] The authors wish to express their appreciation to the management of the Naugatuck Chemical Division, U. S. Rubber Co., for permission to publish these data which were obtained at the Agricultural Chemicals Laboratory, Bethany, Conn.

[2] The active ingredient of Phygon, registered trademark. U. S. Patent 2,302,382 assigned to U. S. Rubber Co.

Copyright, 1950, by Boyce Thompson Institute for Plant Research, Inc.

pressure per square inch. Application was made from a portable boom equipped with four nozzles for each row of potatoes and five nozzles for tomatoes. When used dry, the mixtures were applied at the rate of approximately 50 lb. per acre from a rotary, hand-operated duster. Treatments were applied to single rows (7 plants each) of tomatoes spaced 5 feet apart, and on single 20-foot rows of potatoes. Five replicates were used on tomatoes and six on potatoes. Treatments were applied on July 30, August 9, 22, and 30. During the intervening periods, insects were controlled effectively by separate applications of DDT at the rate of 1 quart of 25 per cent emulsion in 100 gallons of water.

Data on the severity of leaf blight, caused almost exclusively by *Phytophthora infestans*, were taken on August 28 by the visual scoring

TABLE I

COMPOSITION OF EXPERIMENTAL DUST USED IN EXPERIMENTS

Ingredients used	Percentage material in formulations		
	Dust A	Dust B	Dust C
Dichloronaphthoquinone	3.0	3.0	3.0
Sulfur	20.0	—	—
Cubé resin (23.5% rotenone)	3.2	3.2	3.2
DDT	4.0	—	4.0
Celite	10.0	10.0	10.0
Talc	58.8	82.8	78.8
Nacconol NR	1.0	1.0	1.0

method of Horsfall and Barratt (8). The tomato fruit was harvested and weighed when fully ripe on four different dates. The potato tubers were dug on October 17. Those with tuber rot or of substandard size were discarded, and the remainder was weighed.

The measurement of particle size in the dust-spray formulations presented a special problem since there were three or four insoluble components in the mixtures. Direct microscopic examination was not practical so a procedure based upon differential sedimentation followed by chemical analysis was used. A polarographic method (5) that was not interfered with by sulfur, DDT, or rotenone was developed. A sample of the formulation under investigation was suspended in 0.25 per cent Nacconol NR solution and agitated until agglomerates were disintegrated. The suspension was then placed in a 4-liter cylinder and samples were siphoned off at various levels for analysis after different periods of sedimentation. From the data obtained on changes in concentration of dichloronaphthoquinone with time, it was possible to calculate the particle size distribution of the dichloronaphthoquinone by application of Stokes' law. Details of this method have been described elsewhere (5).

A modification of the sedimentation technique was used to separate particles of different sizes for bioassays (2). For these preparations, a sample of technical dichloronaphthoquinone was purified by sublimation and recrystallization from benzene. The lemon yellow crystals (m.p. 190° C.) were ground by two passages through a Raymond pulverizer equipped with a 0.25 mm. herringbone screen. A 200-gram sample was

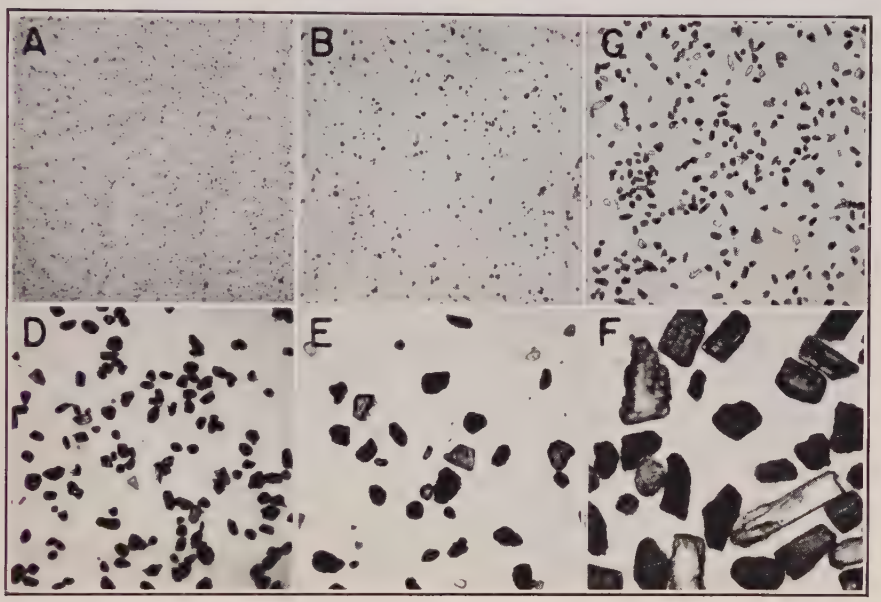


Figure 1. Representative samples of dichloronaphthoquinone from fractions designated as having particles of radii 0.5 to 1.0μ (A), 1.0 to 3.0μ (B), 3.0 to 5.0μ (C), 5.0 to 10.0μ (D), 10 to 20μ (E), >20μ (F). The particles average 0.81, 1.48, 3.57, 8.36, 13.8, and 24.5μ in radius.

pasted in a mortar with 0.25 per cent solution of Nacconol NR in distilled water and then diluted to 4 liters by adding Nacconol solution. After being stirred until all agglomerates disappeared, the suspension was allowed to settle until all particles larger than 5μ radius were calculated to be below the midpoint of the cylinder. The upper half was then siphoned off through a tube with a U bend at the tip to avoid disturbing the lower fractions. These two fractions were resuspended, diluted, and further fractional sedimentations made at different intervals to secure samples that were calculated to have particles with radii of <0.5, 0.5–1.0, 1–3, 3–5, 5–10, 10–20, and >20 microns.

Each sample was analyzed for dichloronaphthoquinone content (3) and adjusted to a concentration of 2000 p.p.m. The mean radius of the particles in each fraction was then determined by counting the number of particles

in a known volume in a Levy blood counting chamber by the method of Wilcoxon and McCallan (13). Although the particles of dichloronaphthoquinone were not spheres (see Fig. 1) as postulated for the application of Stokes' law, the separations were satisfactory. The observed mean radii of the samples agreed well with the calculated sizes and 93 to 98 per cent of the material fell in the specified ranges (2).

The fungitoxicity of the various fractions was determined by assaying their ability to inhibit spore germination on glass slides and to prevent infection of tomato foliage. The glass slide germination test with 7-day-old conidia of *Alternaria oleracea* Milbr. and *Sclerotinia fructicola* (Wint.) Rehm. as recommended by the committee of the American Phytopathological Society (1) was employed. The procedure for evaluating ability to prevent infection of tomato foliage by *Alternaria solani* was essentially that described by McCallan and Wellman (9) with slight modification as described elsewhere (10). The data on ability to protect foliage were considered most applicable to the evaluation of protectant fungicides; so they are emphasized in this paper.

EXPERIMENTAL RESULTS

Three formulations of dichloronaphthoquinone were tested in dosage series as sprays on potatoes in 1946. In addition, another formulation and four dust compositions were tested at a single dosage rate. Conditions were exceptionally favorable for the development of *Phytophthora infestans* which was observed shortly after the second spray. Unsprayed controls were almost completely destroyed by the last of August as can be seen by the data in Table II. At that time only 9 per cent of the foliage remained disease-free as compared to 88 per cent in the plots sprayed with Bordeaux mixture 4-2-50. The dichloronaphthoquinone formulations at rates of 4 to 9 ounces of fungitoxicant per 100 gallons of mixture gave comparable disease control. In general, the dichloronaphthoquinone in the dust-spray formulations was approximately twice as effective as in the standard material. A comparable relationship held at the other dosages. The dusts were not so effective as the sprays but the dust-sprays were definitely superior to Phygon dust at 2 or 5 per cent concentration.

The disease control was reflected in yield of No. 1 tubers. The unsprayed plants yielded 150 bushels per acre. The various treatments increased the yields by 48 to 180 bushels per acre. The dust-sprays were more effective in increasing yields than Phygon at equivalent weights of dichloronaphthoquinone. Plants protected with the lowest dosage (9.6 ounces of technical dichloronaphthoquinone per acre in each application) of either dust-spray yielded as well as those sprayed with 32 ounces of commercial dichloronaphthoquinone. A careful study of the data suggests that the dust-sprays were about twice as effective as commercial dichloronaphthoquinone per unit of fungitoxicant.

The same general relationship was indicated when the materials were applied as dusts. Although quantitative comparisons cannot be made in the absence of a dosage series, formulations A and C as dusts were better than 2 per cent Phygon dust. The data suggest that they were also superior to 5 per cent Phygon dust but the differences are not statistically significant.

TABLE II

Relative Effectiveness of Four Formulations of Dichloronaphthoquinone in Preventing Infection of Potatoes by Phytophthora infestans

Spray treatments applied		Approx. amt. applied, oz./acre	Control of leaf blight, %	Yield of sound tubers, lb.	Calculated yield per acre, bu.
Formulation of chemical	Concn., oz./100 gal.				
Phygon, wettable	32	64	94	24.3	268
	16	32	93	18.9	212
	8	16	85	19.4	226
	4	8	81	16.1	184
Dust-spray (3%) A	480	28.8	96	26.4	300
	320	19.2	91	28.5	331
	160	9.6	89	23.4	265
Dust-spray (3%) B	480	28.8	96	24.6	279
	320	19.2	93	23.7	270
	160	9.6	75	20.8	240
Dust-spray (3%) C	320	19.2	92	24.5	279
Bordeaux-mix. (4-2-50)	—	—	88	21.7	243
Phygon dust (5%)	Dust	40	58	19.6	218
Phygon dust (2%)	Dust	16	50	17.6	198
Dust-spray (3%) A	Dust	24	88	24.9	290
Dust-spray (3%) B	Dust	24	89	21.4	247
Untreated	—	—	9	13.6	153
	—	—	9	12.9	148
Min. sign. diff.—5%				6.3	
Min. sign. diff.—1%				8.3	

Many factors might have contributed to these differences in yield. Although insects, such as leafhoppers, apparently were controlled in all blocks by treatment with DDT emulsion, the supplementary dosages of DDT and rotenone in the dust-sprays may have perfected the control. There is every reason to assume, however, that the major influence on yield was due to disease control because of the close correlation between the two factors.

Comparable data were obtained on tomatoes grown in the same field and treated with the same materials (Table III). The crop was completely

destroyed, as far as all practical purposes were concerned, by *P. infestans*. Foliage infection was lighter than in the potatoes, but fruit rot was almost 100 per cent on unsprayed plants after the first picking. Yield of sound fruit was increased from 1.54 tons per acre to about 10 tons by the better treatments. The general trend was the same as on potatoes and differed only slightly in minor details. Since insects were not observed on the tomatoes, the differences in yield can be attributed to disease control even more certainly than for potatoes. The addition of sulfur to dichloro-

TABLE III

RELATIVE EFFECTIVENESS OF SEVERAL FORMULATIONS OF DICHLORONAPHTHOQUINONE IN PREVENTING PHYTOPHTHORA BLIGHT AND INCREASING YIELDS OF TOMATOES

Spray treatment used, formulation of chemical	Concn., oz./100 gal.	Control of leaf blight, %	Yield of marketable fruit, lb.	Calculated yield per acre, tons
Phygon, wettable	32	94.0	43.5	9.02
	16	92.0	32.8	6.81
	8	76.0	20.1	4.18
Dust-spray (3%) A	480	89.0	50.2	10.42
	320	89.0	34.8	7.21
	160	91.0	18.5	3.84
Dust-spray (3%) B	480	86.0	45.9	9.52
	320	91.0	35.8	7.42
	160	84.0	17.7	3.67
Phygon dust (5%)	Dust	89.0	31.2	6.47
Phygon dust (2%)	Dust	86.0	14.2	2.95
Dust-spray (3%) A	Dust	81.5	25.2	5.24
Dust-spray (3%) B	Dust	76.0	24.1	5.01
None	—	35.0	7.4	1.54
Min. sign. diff. 5%			6.7	
Min. sign. diff. 1%			8.9	

naphthoquinone, as was done in dust-spray A, did not increase its effectiveness against this disease, either on potatoes or tomatoes. The DDT apparently had no appreciable effect on disease control since formulae A, B, and C were almost identical in performance where tested at equal dosages.

The greater effectiveness of dichloronaphthoquinone in dust-spray formulations as compared to undiluted material would indicate that it was more active. However, it might be argued that the various supplements improved the distribution on the leaves, adherence during rainfall, or persistence upon exposure to air and sunlight. In order to eliminate these considerations pertaining to spray deposits, tests were made on potted tomato plants in the greenhouse. Two samples each of dust-spray and Phygon were applied in dosage series to duplicate plants while they

were rotating on a turntable under a spray hood where uniform spray coverage could be obtained. As soon as the spray deposits were dry the plants were exposed to infection by *Alternaria solani*.

The data on blight control, presented in Table IV, show that the two dust-spray samples were superior to Phygon. Inasmuch as the variables of coverage and persistence had been eliminated, these differences must be attributed to innate differences in fungitoxicity. These data show that the two samples of dust-spray provided 95 per cent effective control at 85 and 70 p.p.m. as compared with 132 and 122 p.p.m. of technical dichloro-

TABLE IV

Relative Effectiveness of Dust-Spray and Phygon in Protecting Tomato Foliage from Infection by Alternaria solani

Spray treatment applied	Blight control (%) on plants sprayed at various concns. (p.p.m.) of dichloronaphthoquinone					Estimated ED_{95} value, p.p.m.	Relative dosage ratio
	320	160	80	40	20		
Phygon 120	99.4 100.0	95.5 98.2	85.0 88.6	70.7 61.7	70.7 74.0	132	1.89
Dust-spray 88	100.0 100.0	98.8 99.4	93.7 91.6	74.3 73.4	74.3 71.0	85	1.21
Phygon 142	100.0 100.0	95.8 98.2	91.3 81.3	52.1 55.7	50.3 27.2	122	1.74
Dust-spray 61	99.1 100.0	99.4 100.0	96.4 94.9	89.5 84.1	81.4 80.8	70	1.00

naphthoquinone. Thus, in order to secure equivalent control, 75 to 90 per cent more active ingredients was required than of dichloronaphthoquinone in dust-spray. These data are in good agreement with the field data in Tables II and III; so the differences in field performance may be attributed legitimately to differences in fungitoxicity rather than to some aspect of spray deposition.

SIZE OF PARTICLES IN DIFFERENT FORMULATIONS

Some of the formulations that differed in fungitoxicity were suspended in distilled water and analyzed for particle size distribution by sedimentation rate. Typical data obtained on three compositions are given in Figure 2. Over 21 per cent of the material by weight in technical dichloronaphthoquinone occurred as particles with radii greater than 22μ. Only 25 per cent of the material was in particles less than 5μ. The 3 per cent garden dusts had much better distribution. There was over twice as much dichloronaphthoquinone less than 5μ. Approximately 60 per cent of the particles greater than 22μ had been fragmented in blending the dichloronaphthoquinone into dust-spray.

These observations suggest a ready explanation for most of the differ-

ences in fungitoxicity. If an organic material such as dichloronaphthoquinone operates in the same manner as insoluble copper (6, 7, 12) and sulfur (4, 6, 13) fungicides, the smaller particles would be more effective. Since ability to protect surface depends upon the number of particles, and availability depends upon the surface area exposed to the fungus spore, it is logical to assume that these principles hold for all moderately insoluble fungicides.

The reasons for the smaller particles in dust-spray are of passing inter-

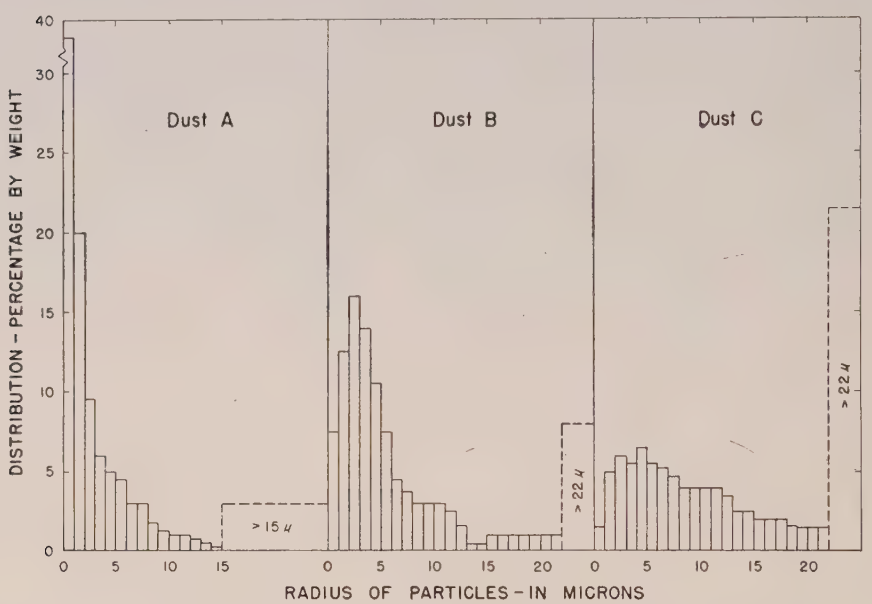


FIGURE 2. Particle size distribution of dichloronaphthoquinone in three formulations: A, 3% in calcium carbonate; B, 3% in dust-spray; C, original sample of dichloronaphthoquinone.

est. Since the diluents may have served as abrasives in the grinding operations, tests were made with several of the common diluents. Talc was found to be more effective than Celite in fragmenting dichloronaphthoquinone (5). Calcium carbonate was the most effective material for reducing particles to a size of less than 1μ radius as can be seen from the data in Figure 2. Almost 40 per cent of the material was in this class and only 3 per cent occurred as particles greater than 15μ radius. The data of others (4, 6, 7, 13) on sulfur and copper suggest that this would be a superior distribution for fungicidal activity, but the question may be raised as to whether such extreme fineness is necessary or even desirable in organic compounds where there is a tendency for the active ingredients to decompose or oxidize upon exposure to the air.

RELATIONSHIP OF PARTICLE SIZE TO FUNGITOXICITY

Seven samples obtained from pure dichloronaphthoquinone by fractional sedimentation (2) were tested for fungicidal ability on glass slides alongside a standard sample of material that contained the entire normal range of particle sizes. Data on the germination of 7-day-old conidia of *Alternaria oleracea* and *Sclerotinia fructicola* in different concentrations of the chemical are presented in Table V. The *A. oleracea* spores were some-

TABLE V

Effect of Dichloronaphthoquinone Particles of Different Sizes on Germination of Spores on Glass Slides

Size of particle, microns	Fungus tested*	Percentage spore germination in various concns. (p.p.m.)					
		31.6	10.0	3.16	1.00	.316	.100
0–0.5	*A.*	—	—	0.0	0.5	94.5	99.0
0–0.5	*S.*	—	—	0.0	0.0	2.0	84.5
0.5–1.0	*A.*	0	0	0	20.0	96.5	100.0
0.5–1.0	*S.*	0	0	0	1.5	6.0	36.5
1–3	*A.*	0	0	0	0	80.0	97.0
1–3	*S.*	0	0	0	0	0	9.0
3–5	*A.*	0	0	0	0	94.0	97.0
3–5	*S.*	0	0	0	0	0	5.0
5–10	*A.*	0	0	0	38.0	97.0	99.0
5–10	*S.*	0	2.0	0	2.0	3.0	70.5
10–20	*A.*	0	0	90.5	90.5	98.0	100.0
10–20	*S.*	0	0	8.0	31.5	80.5	82.5
Over 20	*A.*	22.0	92.5	99.5	98.5	99.5	97.0
Over 20	*S.*	0	0	74.0	86.5	84.5	79.0
Standard	*A.*	0	0	0	9.0	97.5	99.5
Standard	*S.*	0	0	0	0	7.5	64.5
Untreated	*A.*						96**
Untreated	*S.*						92

* *A—Alternaria; S—Sclerotinia.*

** Test samples corrected to 100 by counting extra spores for non-viable members.

what more resistant to dichloronaphthoquinone than usual in this test. The data, however, are in sufficiently close agreement with *S. fructicola* to leave little doubt as to the relative values of the various samples.

The standard sample had an ED50 of slightly more than 0.1 p.p.m. which is in keeping with the figure of about 0.04 p.p.m. usually obtained in such tests. Comparable data were obtained on the 5 to 10μ particles. Particles larger than these were one-fifth to one-thirtieth as effective and those of 1 to 3μ or 3 to 5μ radius were about three times as effective. The particles that were less than 1μ in radius were less effective than the 1 to 3μ

class. The reason for this unanticipated result is not obvious unless the smaller particles were inactivated by the orange juice added as a spore stimulant.

The ability of these seven fractions and the standard to protect tomato foliage from infection by *A. solani* was tested. Three small, potted plants were sprayed with each material at concentrations of 400, 200, 100, 50, 25, 12.5, and 6.25 p.p.m. Due to the large number of plants involved it was impossible to expose all of them to infection at the same time in the incubation chamber available for the test. The plants were divided into three sets, each including a plant from each treatment and inoculated on successive days so 48 hours elapsed from the time of spraying until the last

TABLE VI

EFFECTIVENESS OF DIFFERENT SIZED PARTICLES IN PREVENTING INFECTION OF TOMATOES BY ALTERNARIA SOLANI UNDER GREENHOUSE CONDITIONS

Radius of particles in sample, microns	Percentage disease control* at various concns. (p.p.m.)							ED95 value, p.p.m.
	400	200	100	50	25	12.5	6.2	
<0.5	—	100.0	99.1	93.2	85.1	12.2	19.2	52
0.5– 1.0	100.0	99.6	98.7	92.3	73.4	1.3	0.7	64
1.0– 3.0	99.6	99.5	97.7	91.7	52.3	9.4	4.8	89
3.0– 5.0	98.6	96.1	93.2	83.3	51.3	5.6	21.4	157
5.0–10.0	95.5	60.8	42.5	20.1	0.0	5.8	22.0	445
10.0–20.0	73.2	12.8	7.0	18.1	2.2	8.3	0.0	870**
>20	33.0	10.3	5.1	9.5	0.0	5.5	0.9	2200**
Standard	98.8	98.5	92.8	83.0	41.3	9.3	21.2	135

* Mean numbers of lesions in unprotected controls in the three different sets were 523, 1104, 488.

** Approximate figure obtained by extrapolation.

set was exposed to fungus spores. The three sets gave comparable results with only minor variation.

The mean percentages of blight control in these triplicate tests are presented in Table VI. These data yield very satisfactory straight line dosage curves when plotted on logarithmic-probability paper. The lines are closely parallel, with the exception of the 3 to 5μ sample, thereby indicating that the mechanism of killing was identical and that the various sized particles differ only in their availability to the fungus spores.

There was a progressive loss in fungitoxicity with increasing size of particle so that the ED95 values ranged from 52 p.p.m. through 64, 89, 157, 445, 870 to about 2200 p.p.m. The standard sample had an ED95 of 135 p.p.m. which agreed most closely to the 3 to 5μ class. The relationship of particle size and ED95 values held throughout the range of particle sizes with no tendency for particles $<1\mu$ to be less effective than 1 to 3μ as was observed in the spore germination tests on glass slides.

These data offer evidence of why the samples with different size distributions (Fig. 2) differed in field and laboratory performance. The 20 per cent of coarse particles ($>20\mu$) in technical dichloronaphthoquinone contributes very little to fungitoxicity. By converting most of them to the smaller class by the abrasive action of talc in dust-spray the specific activity was increased several-fold.

The foregoing test was repeated in its entirety with samples derived from another refined lot of dichloronaphthoquinone. The data were in fair agreement with those presented in Table VI. The major discrepancy was that particles less than 1μ had ED95 values of about 36 to 40 p.p.m. instead of 52 p.p.m.

TABLE VII

Relative Fungitoxicity of Intermediate Sized Particles of Dichloronaphthoquinone Derived from Large and Small Particles by Recrystallization from Acetone Solution

Type of particle tested		Percentage blight control* at various concns. (p.p.m.)						Estim. ED95 value, p.p.m.
Source of particles	Treatment after centrifugation	800	320	160	80	40	20	
Small, $<1\mu$	Resuspended	—	99.9	99.4	97.8	87.3	72.2	60
Small, $<1\mu$	Recrystallized	—	99.5	97.0	86.3	73.9	80.5	115
Large, $5–10\mu$	Recrystallized	—	99.6	96.9	91.2	84.2	87.1	110
Large, $5–10\mu$	Resuspended	98.7	95.9	86.1	79.5	70.2	—	365

* Sample with resuspended $5–10\mu$ particles was tested at 800, 400, 200, 100, and 50 p.p.m. and data recorded in the nearest proximate column. Data based on an average of 861.5 lesions on 8 unprotected plants.

One further experiment was made on particles of different sizes to eliminate doubt as to the relative purity of large and small sized fractions. It is evident that if samples of $<1\mu$ and 5 to 10μ particles, for example, were reduced to a common size, they should be of identical fungitoxicity if they were of identical composition. Such a test was made by taking duplicate 200 ml. aliquots of each sample, centrifuging until all particles were sedimented, discarding the clear supernatant fluid, dissolving one aliquot of each sample in acetone, adding 1 ml. of Emulfor EL and emulsifying in distilled water by agitation in a Waring blender for 10 minutes. The second aliquot was resuspended in water with 1 ml. of Emulfor EL by agitation for 10 minutes without first dissolving it.

These four samples were tested in dosage series on quadruplicate tomato plants for ability to protect foliage. The data presented in Table VII show that the particles of $<1\mu$ and 5 to 10μ radii had ED95 values of 60 and 365 p.p.m., figures which are in fair agreement with preceding data (Table VI). The aliquots that had been dissolved in acetone were as nearly identical in fungitoxicity as would be expected with ED95 values of 110

and 115 p.p.m. When the data on disease control were plotted on logarithmic-probability paper to obtain these values, good straight line dosage curves were obtained except for the lower dosages. The lower dosages gave slightly better disease control than would have been anticipated, presumably because of residual chemical from the preceding series which was incompletely washed from the spray gun. The four samples had curves with similar slopes, indicating that the mechanism of fungitoxicity probably was identical irrespective of particle size.

DISCUSSION

The data obtained with dichloronaphthoquinone in these experiments are in good agreement with those recorded (4, 6, 7, 12, 13) for sulfur and copper fungicides. Apparently the same physical laws operate for organic compounds as for inorganic materials. The smaller particles are more readily available to the fungus spore because of the large number per unit weight or their greater specific surface.

Dichloronaphthoquinone has been recognized as one of the more potent organic fungicides discovered but it has been somewhat expensive to manufacture and it has caused mild injury to some crops at the concentrations recommended. By taking advantage of the data described herein, it would be possible to blend the material with a suitable diluent and obtain a more favorable particle size distribution. The type of diluent had to be chosen from those materials that resulted in a particle size predominantly in the range of 1 to 10μ radius since extreme fineness was considered detrimental to persistence in an effective protective layer for long periods of time. The modified formulation (Phygon XL) was about twice as effective in protecting foliage as the more coarse material. Data have been published on apple scab (11) showing that $\frac{3}{8}$ lb. of material in this formulation is approximately as effective as $\frac{3}{4}$ lb. of Phygon per 100 gallons of spray mixture. This change in physical properties, therefore, was sufficient to make the organic compound fully competitive with the cheaper inorganic spray materials. At the same time it reduced the density of spray deposit so that there was less likelihood of injuring the crop plants.

These data on particle size focus attention on one of the more serious errors in ordinary screening tests for protective fungicides. It is obvious that comparative ED_{50} values on glass slides (Table V) or ED_{95} values on tomato foliage (Table VI) of a series of organic compounds is only relative and not absolute as long as tests are made without regulation of particle size to a common unit.

The major consolation we have at present is that no really good fungicides have been discovered by other tests after being rejected on the basis of these standard laboratory tests. The tests must still be considered as reliable, practical screening techniques even though they are deficient in

fundamental significance. Those who use these techniques, however, are obligated to conduct them in such a fashion as to obtain reproducible results. This calls for grinding all test materials to a common size with a suitable surface-active agent, a rather impractical application; or dissolving them in a water miscible solvent and precipitating them in aqueous suspension with suitable emulsifying agents. The latter process is more expedient and each material probably precipitates consistently in about the same particle size range.

There is no perfect method for solving the dilemma presented by particle size effects in making comparative tests of fungicides. Probably the ideal for the glass slide test would be to base ED_{50} on mols of dissolved materials which would necessitate starting all tests at a maximum dosage of saturation at 70° F. If particles of undissolved material are available, the rate of solubilization as the spore absorbs the fungicides may become a dominating factor in determining effective kill.

The chemical analyses for quality control are often supplemented by fungicide tests. This is done because minor differences in chemical composition which are not detected by customary tests may influence biological performance. Often, in the rush to complete bioassays before samples are shipped, tests are started on crude batches before grinding. It is obvious that errors in grinding may be as serious as deviations in synthesis; so some auxiliary test, such as sedimentation rate, should be made to be certain that the particle size range is satisfactory. If this is done full confidence could be placed in a tomato foliage or slide test in which the crude product had been completely dissolved in acetone or other non-toxic solvent and precipitated in aqueous suspension at a common particle size.

Theoretically, it should be possible to predict field performance of samples of dichloronaphthoquinone that differ in particle size distribution. By weighting each class of particles according to its ED_{95} value and multiplying by its proportional occurrence, a mean value of performance for the sample can be predicted. Additional data on the reliability of such calculations will be presented in a subsequent paper. The present data, however, are adequate to deduce that a major influence in determining the potency of dust-spray formulations was the finer particles of dichloronaphthoquinone produced during blending operations. The data do not, however, eliminate the possibility of synergism or reaction between dichloronaphthoquinone and rotenone. There was no suggestion of synergism from sulfur or DDT in the field trials.

SUMMARY

1. Dichloronaphthoquinone formulated in special all-purpose garden dusts with insecticides and sulfur was more effective than pure chemical

in preventing the late blight of tomatoes and potatoes caused by *Phytophthora infestans* under field conditions.

2. The special formulations were also more effective in preventing early blight of tomato caused by *Alternaria solani* in greenhouse experiments.

3. The dichloronaphthoquinone particles in the special formulations were smaller than in the parent material. Experiments showed that particle size could be altered by grinding with different types of diluents.

4. There is a direct correlation between the effectiveness of material in protecting foliage and the particle size at time of deposition. About forty times as much material with a particle size of 20μ is required for comparable disease control as of 1μ particles. There was some evidence that particles $<1\mu$ in radius were less effective on glass slides than those 1 to 3μ.

5. The results of laboratory screening tests depend, in part, upon the size of particles used. This factor should be taken into consideration in preparing samples for testing.

LITERATURE CITED

1. AMERICAN PHYTOPATHOLOGICAL SOCIETY. COMMITTEE ON STANDARDIZATION OF FUNGICIDAL TESTS. Test tube dilution technique for use with the slide-germination method of evaluating protectant fungicides. Phytopath. **37**: 354–356. 1947.
2. BURCHFIELD, H. P., DELORA K. GULLSTROM, and G. L. MCNEW. Agricultural dusts. Preparation of dusts of uniform particle size by fractional sedimentation. Analyt. Chem. **20**: 1168–1173. 1948.
3. BURCHFIELD, H. P., and G. L. MCNEW. The colorimetric determination of 2,3-dichloro-1,4-naphthoquinone on seed. Phytopath. **38**: 665–669. 1948.
4. FEICHTMEIR, EDMUND F. The effect of particle size and solubility of sulfur in carbon disulfide upon its toxicity to fungi. Phytopath. **39**: 605–615. 1949.
5. GULLSTROM, DELORA K., and H. P. BURCHFIELD. Agricultural dusts. Determination of particle size distribution. Analyt. Chem. **20**: 1174–1177. 1948.
6. HAMILTON, J. M., D. H. PALMITER, and G. L. MACK. Particle size of sulphur and copper fungicides in relation to apple scab and cedar-apple rust control. Phytopath. **33**: 533–550. 1943.
7. HEUBERGER, JOHN W., and JAMES G. HORSFALL. Relation of particle size and color to fungicidal and protective value of cuprous oxides. Phytopath. **29**: 303–321. 1939.
8. HORSFALL, J. G., and R. W. BARRATT. An improved grading system for measuring plant diseases. Phytopath. **35**: 655. 1945. (Abstract)
9. MCCALLAN, S. E. A., and R. H. WELLMAN. A greenhouse method of evaluating fungicides by means of tomato foliage diseases. Contrib. Boyce Thompson Inst. **13**: 93–134. 1943.
10. MCNEW, GEORGE L., and NORMAN K. SUNDHOLM. The fungicidal activity of substituted pyrazoles and related compounds. Phytopath. **39**: 721–751. 1949.
11. SCHOENE, D. L., H. DOUGLAS TATE, and T. W. BRASFIELD. Use of quinones as fungicides. Agric. Chem. **4**(11): 24–27, 73, 75–77. 1949.
12. TWENTYMAN, R. L. Experiments on the control of "stinking" smut or bunt. Part 2. Tests of the dry copper powders. Jour. Dept. Agric. Victoria **29**: 235–248. 1931.
13. WILCOXON, FRANK, and S. E. A. MCCALLAN. The fungicidal action of sulphur. III. Physical factors affecting the efficiency of dusts. Contrib. Boyce Thompson Inst. **3**: 509–528. 1931.

FLOWERING AND OTHER RESPONSES INDUCED IN PIQUERIA TRINERVIA WITH PHOTOPERIODIC TREATMENT

P. W. Zimmerman and Julie Kjennerud[1]

Considerable evidence has been developed to show that natural plant growth regulators control root, bud, and flower formation of plants. There is also evidence that the influence involved is of a chemical nature. Both natural and synthetic root-inducing substances are well known. However, neither natural nor synthetic bud- and flower-inducing substances have been chemically identified. Short day plants, however, afford good material to indicate the presence of a flower-inducing influence which appears to be of a chemical nature.

The common stevia (*Piqueria trinervia* Cav.), of the florists' trade, is a composite which flowers only during the short days of fall and winter. The response of this species to photoperiodic treatment has not been previously reported. The purpose of this report is to record the requirements for induction of flower buds and some of the associated responses when stevia is given various photoperiodic treatments. Of special interest are the production of terminal inflorescence only, localization of perception, lack of translocation of the stimulus, and the variation of these responses from those of other species.

METHODS AND MATERIAL

Stevia plants were propagated from cuttings and grown in four-inch clay pots. When the young stems were pruned properly two or more branches could be made to grow on the same plant. In order to regulate light exposure individual branches or leaves can be covered with black sateen cloth or aluminum foil. The cloth was used in 1947, 1948, and in the early experiments in 1950. Aluminum foil has been used in all recent experiments. The foil can be pressed into position and need not be tied. Also the foil is entirely impervious to light which is not true of cloth. The temperature under the foil when the sun was shining was only a few degrees above that of the surrounding air. Under black cloth the difference was sometimes 25° F.

In some experiments the plants were transferred from the greenhouse to the dark room for 16-hour periods. The temperature of the dark rooms ranged from 35° F. to 50° F. This gave an opportunity to study the effect of different temperatures during the dark periods. Artificial light was used at night to prevent flowering during the short winter days. This method made possible the desired variation in photoperiods throughout the entire

[1] Fellow, sponsored by the American Association of University Women.

Copyright, 1951, by Boyce Thompson Institute for Plant Research, Inc.

year. Where photoperiodic treatments lasted for more than five days during 1950, capping or transfers to the dark room were interrupted for two days over the week-end. No attempt was made to control temperature completely except when the plants were held in dark rooms. The greenhouse temperature during the winter months was usually 65° to 70° F. at night but varied during the day when the sun shone.

EXPERIMENTAL RESULTS

Requirements for flower bud initiation and vegetative branching. The first attempts to cause flowering of stevia by shortening the photoperiods were made in 1947. The plants were induced to flower by capping the leafy stem tips with black sateen cloth from 5:00 p.m. to 8:30 a.m. This provided a photoperiod of 8½ hours. The capping started on October 14 and was continued for 49 days though it was later found that a few long dark periods would have sufficed.

Flowering of terminal shoots or individual axillary branches was induced by capping with aluminum foil from 4:30 p.m. to 8:30 a.m., beginning April 11. Responses induced by this method are illustrated in Figure 1. The capping was interrupted for two or three periods over the week-ends between April 11 and May 8 when the capping was stopped. It was later found that the week-end interruptions made little or no difference since the first four or five long dark periods were sufficient to induce flowering. The figure shows flowering of one large branch which had been capped to compare with the control branch on the left. The same figure (right) shows one small branch induced to flower by capping with foil for the same periods mentioned above.

The absolute minimum requirements for dark periods have not been determined. The results of tests during these experiments, however, showed that flower buds were initiated by two 16-hour dark periods with one intermittent 8-hour photoperiod. Since the capping treatments started at 4:30 p.m. and ended at 8:30 a.m. the photoperiods before the first dark period and after the last dark period were shortened by approximately three hours. The short photoperiods at the beginning and end of the treatments did not appear to affect the initiation of flower buds. The experiments involving the attempts to determine the minimum requirements for dark periods to induce flower buds were started during the long days of late spring and repeated during September. The results were the same in both tests. The experiments with stevia involved one to five 16-hour dark periods with intermittent 8-hour photoperiods. One 16-hour dark period did not initiate flower buds and the plants continued in what appeared to be a normal vegetative condition. Initiation of flower buds occurred with two or more dark period treatments. Though the flower buds could not be seen with the naked eye for approximately 15 to 20 days, development

FIGURE 1. Effect of capping stevia plants with aluminum foil 17 times for 16 hours each with 8 hours intermittent photoperiods. Left, plant with two main shoots showing flowering on right branch due to capping; right, plant showing flowering of one small branch following capping.

continued even though the plants were grown under long photoperiods.

Flowering did not occur when the length of the dark periods was increased to 22 hours and the photoperiods reduced to two hours. When the photoperiods were reduced to four hours with corresponding 20-hour dark periods flower buds were initiated as usual. No tests were made with 3-hour photoperiods.

As shown in Table I a few flowers were produced by two 16-hour dark periods and the number increased with the number of dark periods up to five. The flowers were not carefully counted beyond the five dark period treatments but the appearance of plants treated for longer periods of time indicates that the number of flowers can be increased with continued treatments. With long continued dark period treatments vegetative growth ceased and the terminal parts of all branches formed flowers (Fig. 1).

TABLE I

TOTAL NUMBER OF INFLORESCENCES ON MAIN STEM OF PLANTS 95 DAYS AFTER VARIOUS LONG, DARK PERIOD TREATMENTS

No. of long, dark periods	No. of inflorescences	Av. No. for two plants
1	Vegetative	0
1	Vegetative	
2	94	109
2	123	
3	220	167
3	114	
4	220	205
4	189	
5	159	237
5	314	

With five or fewer dark periods the shoots developed some vegetative branches in addition to flowers (Fig. 2). However, when the branches were fully stimulated and flowering occurred there was no reversion to the vegetative stage. Stevia differs in this respect from several other species with terminal flowers such as *Salvia splendens*, cosmos, and poinsettia as reported by Roberts and Struckmeyer (6). It can be seen in the illustrations that the main shoot as well as axillary branches always produce terminal flowers. This is associated with a decrease in the rate of elongation of the shoot and an increase in branching (Fig. 2).

Stevia plants that had been given photoperiodic treatment insufficient for complete change to reproductive growth often showed a qualitative difference in the two shoots at the same node of an axillary branch, as one of the shoots might be vegetative, the other one reproductive. This was associated with a difference in size of the leaf where the shoot was produced, the vegetative shoot growing in the axil of a larger leaf while the flowering shoot appeared in the axil of a much smaller leaf. In cases where the shoots were strictly reproductive the leaves at their base were reduced to narrow bract-like structures 4 to 5 mm. in length.

Single long dark periods without short photoperiods were ineffective for initiating flower buds. Such test periods lasted from 40 to 232 hours. It cannot be said, therefore, that the light periods are not important. In another test involving a 32-hour dark period the plants were capped at 8:00 a.m. after they had received about three hours of natural light; the caps were removed at 4:00 p.m. the following day which permitted

FIGURE 2. Effect of five dark periods 79 days after capping one stevia branch with foil. The treated shoot (right) shows mixed flowering and non-flowering branches and decrease in elongation in contrast with control (left).

approximately three hours of light in the evening. Therefore, two 3-hour light periods with one long intermittent dark period did not induce flower buds. Two long dark periods interrupted by one, two, or three intermittent natural nights in late May did not initiate flower buds. Also, in this case no vegetative branching occurred.

Localization of perception and translocation of the influence. Flowering occurred only on the capped portion of branches. The first flowers came on the terminal part of the branch and there was no translocation of the "flowering influence" beyond the capped parts.

In some tests the lower portion of a branchless stem 2 to 4 inches below the tip was covered for five periods of 16 hours each with intermittent 8-hour light periods. Other covered stems had axillary branches in the lower part when the experiments started. In such cases flowering occurred on the branches, but not on the terminal part above the covered region. In some experiments, however, where the tip was defoliated a few flowers appeared above the covered part.

Capping of shoots always involved four or more pairs of leaves. The uppermost leaves were barely visible when the experiment started. These treatments always caused flowering at the tip even though the largest of the covered leaves was not fully developed. No flowers occurred below the capped part. When the entire plant was given short day treatments the response was more or less systemic but when only a terminal shoot was capped with aluminum foil the response was local. There was no apparent translocation of the stimulus from a treated branch to an opposite control branch.

Receptor organs. Initiation of flower buds was induced by covering a single leaf of a shoot. When the second, third, or fourth leaf below the tip was covered with foil for nine 16-hour dark periods with 8-hour intermittent photoperiods, flowering occurred. This proves that the leaf is the receptor organ and since the flower buds were initiated at the tip of the shoot the "flowering influence" was translocated up to the growing point. There was no indication that the influence ever moved downward. The age of the leaf or the distance the stimulus could be translocated was important. Covering the fifth leaf below the tip did not cause flowering during these experiments. There still remains a possibility that covering both leaves at the fifth node might induce flowering.

Temperature effects during treatment. The effects of low temperature during the dark periods were tested by placing plants in dark rooms at 35°, 40°, and 50° F. These plants were compared with others capped with aluminum foil in the greenhouse held at 65° F. or higher.

In general, results showed that induction of flowers does not take place when plants are in the dark at 35° F. and that the number of flowers per plant increased with temperature up to 70° F. Also the number of flowers

per plant increased with the number of 16-hour dark periods up to nine which was the longest time any of the plants were subjected to photoperiods during this experiment. Five dark periods were near the minimum required at 50° F. Nine dark periods induced flower buds at 40° F. Flowering occurred on the plants at high temperature within 21 days. Longer time was required for plants treated at 50° F. and still longer for those treated at 40° F. Table II shows the number of flowers produced under the different temperature conditions. Figure 3 shows the effect of dark periods and various temperatures on the branching habit and number of flowers induced.

Influence of photoperiods on branching habit. Branching of stevia has in most cases been associated in some way with induction of flower buds. This response is illustrated in Figure 2. However, it has been possible to induce branching without initiating flower buds. This was done with black cloth not entirely impervious to light. It is not clear whether branching without associated flowering is due to a low magnitude of the stimulus or whether two or more different influences are involved. In general, stevia shoots exhibit a strong apical dominance. When the tip is removed two strong shoots grow from the first node and two weaker ones from the second node. These shoots prevent growth from nodes farther down on the stem. Under natural conditions the plants produce many shoots preceding the flowering period in December. Under extra light, however, the development of axillary shoots ceased when they had reached a length of $\frac{1}{4}$ to $\frac{1}{2}$ inch.

TABLE II

EFFECTS OF TEMPERATURE DURING DARK PERIODS ON NUMBER OF BUDS AND INFLORESCENCES PER PLANT 78 DAYS AFTER TREATMENT STARTED

Temperature, degrees F.	No. of buds and inflorescences after 5 or 9 dark treatments	
	5	9
35	0	0
40	0	10
50	3	150
65–70	438	320

Capping for five nights of four nodes below a point about two inches under the tip caused neither branching nor flowering when the rest of the plant was kept in long days. However, if the long day tip was defoliated, branching was evident after about 14 days both in the short day part and at the defoliated tip (Fig. 4). With the tip above the capped part held in continuous darkness during treatment and for the following 7 days, branching occurred in the short day part only and not at the tip, regardless of whether the tip was defoliated or not.

FIGURE 3. Effect of temperature on flowering of stevia while exposed to five and nine dark periods. A. Plants treated while at 50° F. for five periods (left) and nine periods (right). B. Treatment same as "A" except temperature was 40° F. C. Plants given nine dark periods while at different temperatures; left to right, greenhouse temperature (65° to 75° F.), 50° F., 40° F., and 35° F. No flowers were induced at 35° F.

DISCUSSION

Among the short day plants the Biloxi soybean (*Glycine max* Merr.) and the cocklebur (*Xanthium canadense* Mill.) have been the subject of numerous investigations concerning their responses to photoperiodic treatment. Though both of them are short day plants they differ from each other in several respects; as, for example, their minimum requirements

FIGURE 4. Effect of defoliating the terminal five nodes and covering the stem below as indicated by arrows. Left, terminal nodes not defoliated; right, terminal nodes were defoliated. Note induced branching.

for photoinductive treatment, the location of the meristem that first changes from vegetative to reproductive growth, and the readiness with which the stimulus is translocated within the plant.

Initiation of flowers in the Biloxi soybean may result under ideal conditions from two short photoperiods (5); in the cocklebur from one, or even from one single long dark period unaccompanied by a short photoperiod

(3, 4). Also in the stevia one short photoperiod will cause flower formation, provided the photoperiod is not too short.

A soybean, produced under long day conditions, then subjected to two to four short photoperiods and thereafter returned to long days, will form a few flower primordia in the axil of leaves that are third or fourth from the tip and then resume the production of vegetative structures. The axils where the change takes place have newly organized meristems that have not yet differentiated the primorida of any vegetative leaf. With prolonged short day treatment the apical meristem of the main axis may be caused to form flower primordia, but remains intact and does not change into flowers, even if it discontinues the formation of new structures (4, 5).

With cocklebur the first recognizable effect is an enlargement of the terminal meristem itself, which quickly develops into male flowers. Male

TABLE III

AVERAGE NUMBER OF FLOWERS PRODUCED ON EACH NODE OF STEVIA PLANTS RECEIVING DIFFERENT NUMBER OF DARK PERIODS; COUNTS TAKEN 95 DAYS AFTER TREATMENT STARTED

Location of node below the tip	No. of flowers after 2, 3, 4, and 5 dark treatments			
	2	3	4	5
1	44	48	77	46
2	29	61	46	39
3	0	35	13	35
4	0	0	6	41
5	0	0	50	65
6	36	23	13	11
7	0	0	0	0
Total	109	167	205	237

inflorescences are also formed on axillary buds but only by meristems that have already formed primordia of vegetative leaves. The female inflorescences normally arise from axillary meristems that have not formed vegetative leaf primorida (4).

In the stevia there is an indication that with few photoperiods flower primordia are formed only from meristems that have differentiated vegetative structures. The inflorescences are always terminal, and when the photoperiodic treatment is near the minimum for flower induction the distribution of flowers at the different nodes seems to follow a certain pattern. As can be seen from Table III the plants that received two or three long dark periods had some nodes where the axillary shoots were strictly vegetative. The next node below had flowers, while no flowers appeared lower down. Further, the uppermost of the nodes with vegetative shoots was the last one at which the leaves reached full normal size. These effects also were observed on plants exposed to low temperature during the dark

periods. By examination of a vegetative shoot under a dissection microscope usually two pairs of leaves are found above the last one that can be seen with the naked eye. In most cases after photoperiodic treatment no more leaves were formed beyond these pairs, or at least such leaves did not reach the full size. The nodes mentioned above were the ones that had the longest axillary shoots. With more long dark periods also these shoots developed flowers. This may be an indication that an axillary meristem must reach a certain age before it is able to form flower primordia. Thus the axillary meristems at the single low node where flowers developed would be

FIGURE 5. Effect of covering different parts of cocklebur shoots. Right, entire plant flowered after right branch was covered for eight 16-hour dark periods; left, one branch (right) only flowered after one leaf (second from tip) was covered for eight dark periods.

advanced enough to respond to the stimuli while the ones above were too young. However, they would come to the right stage during prolonged treatments. There is also a possibility that the increased amount of stimulus formed during the longer treatment may cause induction also in the younger meristems, similar to what happens in the soybean under the same conditions.

In cocklebur the stimulus is easily transmitted both up and down the stem and from a short day branch to a non-treated branch on the same plant (3). When *Xanthium* was tested by the present authors, it was found that if one entire branch was covered, the influence became systemic,

extending to other branches. If, however, one leaf was covered, the influence did not extend beyond the branch holding the treated leaf (Fig. 5). In the soybean transfer to a non-treated branch does not take place unless the receptor branch is defoliated (1). If the lower part of a Klondike cosmos (*Cosmos sulphureus* Cav.) plant is kept under short days, translocation of the flowering stimulus to the upper part can take place if this part is kept in continuous darkness (2). In stevia, translocation to a long day branch was never observed, not even in a few cases where the receptor branch was defoliated. Nor did a branch change to the flowering stage by being kept in continuous darkness during five nights of photoperiodic treatment of a short day branch. Translocation up or down the stem did not seem to take place in general except perhaps across very short distances. When the fourth leaf below the tip was covered to afford five dark periods the flowering stimulus was transmitted to the terminal growing point. When a whole plant was given short day treatment, flowers formed only at the stem terminal and in the axils of the very young leaves which were not even developed to half of full size at time of treatment. The small axillary shoots in the lower part remained vegetative and did not elongate. Larger axillary shoots near the base of the plant flowered if they were given short days with the rest of the plant. It was assumed, however, that these shoots were induced by their own leaves and did not receive the stimulus from the main stem.

Branching in the tip region was usually the first sign of macroscopic response to short day treatment of stevia. This effect may be related with changes in the apical meristem which is a consequence of the treatment and reduces the apical dominance. However, branching was not always correlated with flowering as branching sometimes was the only response to short day treatment. When the treatment was inadequate for flowering, branching sometimes occurred after which the tip continued vegetative development without any further branching. In a single case branching even took place at a few nodes on a control plant, kept exclusively on long days. As stated earlier, there may be considerable relationship between apical dominance and branching especially when the photoperiodic stimulus is of a magnitude too low for the induction of flower primordia.

SUMMARY

Stevia plants were given photoperiodic treatments by placing in dark rooms, capping with black cloth, or covering with aluminum foil. In most cases the dark periods were 16 hours long, lasting from 4:30 p.m. until 8:30 a.m.

Flower induction occurred from two or more 16-hour dark treatments with intermittent 8-hour light periods. Floral development persisted under long day conditions after the treatments were stopped. The number of

inflorescences increased with the number of long dark periods while the development of vegetative structures on flowering branches decreased under the same conditions.

The response to short day conditions was local as shown by the fact that only the treated branches of the plants produced flowers. There was no apparent translocation of the stimulus to non-treated branches. The stimulus, however, was transmitted from the second, third or fourth leaf below the tip to the growing point above.

The temperature prevailing during the dark periods was found to have a strong influence on initiation of flower primordia. At 35° F. the plants remained vegetative even after nine long dark periods; at 40° F. five dark periods were ineffective while nine caused a few flowers to be initiated; at 50° F. five long dark periods were near the minimum, and the number of flowers increased with the number of treatments up to nine.

Branching of the terminal region always occurred when the plants flowered. However, branching was not always associated with flowering. This was probably due to inadequate photoperiodic treatment, and the response suggests that more than one influence is involved.

The responses of stevia were discussed and compared with those of other species.

LITERATURE CITED

1. BORTHWICK, H. A., and M. W. PARKER. Photoperiodic perception in Biloxi soy beans. Bot. Gaz. **100**: 374–387. 1938.
2. GARNER, W. W., and H. A. ALLARD. Localization of the response in plants to relative length of day and night. Jour. Agric. Res. **31**: 555–566. 1925.
3. HAMNER, KARL C., and JAMES BONNER. Photoperiodism in relation to hormones as factors in floral initiation and development. Bot. Gaz. **100**: 388–431. 1938.
4. PARKER, M. W., and H. A. BORTHWICK. Influence of light on plant growth. Ann. Rev. Plant Physiol. **1**: 43–58. 1950.
5. PARKER, M. W., S. B. HENDRICKS, H. A. BORTHWICK, and N. J. SCULLY. Action spectrum for the photoperiodic control of floral initiation of short-day plants. Bot. Gaz. **108**: 1–26. 1946.
6. ROBERTS, R. H., and ESTHER STRUCKMEYER. Anatomical and histological changes in relation to vernalization and photoperiodism. *In* Murneek, A. E., and R. O. Whyte *et al.* Vernalization and photoperiodism (A symposium). Pp. 91–100. Chronica Botanica Co., Waltham, Mass. 1948.

HERBICIDAL PROPERTIES OF SODIUM 2,4-DICHLOROPHENOXYETHYL SULFATE

LAWRENCE J. KING, J. A. LAMBRECH,[1] AND THOMAS P. FINN

INTRODUCTION

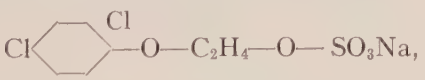
In controlling weeds, particularly annual weeds in cultivated soils, a logical approach has been the application of chemicals to the soil surface to destroy germinating weed seeds. A number of chemicals have been used for this purpose but the growth-regulating chemicals appear to have first been used by Slade, Templeman, and Sexton in 1945 (1, 20). Following the discovery of the growth-regulating properties of 2,4-D by Zimmerman and Hitchcock in 1942 (24) widespread interest developed in this and related compounds. In 1946 three groups of workers (2, 9, 17) demonstrated that 2,4-D prevents the growth of germinating weed seeds when applied to the soil, and since that time many workers have investigated the feasibility of its use on crops in this manner. One of the major limitations of 2,4-D for this purpose is that it may cause serious damage to emerged or emergent crop plants since it is readily adsorbed through leaf surfaces as well as through the roots. Another handicap in its use is the hazard to nearby sensitive plants from drifting spray.

This paper is concerned with sodium 2,4-dichlorophenoxyethyl sulfate,[2] an effective new chemical for use as a soil treatment, which has the unique property of being non-injurious to plants when sprayed or dusted directly on the foliage at the concentrations that will kill seedlings in the soil. Therefore, injury by contact with foliage, either to crops in fields being treated, or in adjoining fields as a result of drift, is minimized.

MATERIALS AND METHODS

PHYSICAL PROPERTIES AND TOXICOLOGY

Sodium 2,4-dichlorophenoxyethyl sulfate,



is a stable, non-volatile, white, crystalline solid which melts at 170° C. It is readily soluble up to 25 per cent by weight in distilled water at room

[1] Research chemist, South Charleston, West Virginia Laboratory, Union Carbide and Carbon Chemicals Corporation. Grateful acknowledgment is given to Dr. R. H. Wellman and to Dr. L. P. Miller for assistance and advice during the course of these investigations.

[2] This chemical has been distributed to a number of investigators as Experimental Herbicide 1 by Carbide and Carbon Chemicals Division, Union Carbide and Carbon Corporation, 30 East 42nd Street, New York 17, N. Y.

temperature. It is sufficiently soluble in hard water, containing 260 p.p.m. of calcium carbonate, to make a 4 per cent solution.

The LD_{50} of sodium 2,4-dichlorophenoxyethyl sulfate determined by single oral doses to rats is 1.4 g. per kilogram of body weight; this is a moderate acute oral toxicity. In the 4-hour rabbit belly skin irritation test a 10 per cent aqueous solution gave a highly irritative action. A 1 per cent aqueous solution on the rabbit eye produced no harmful effects; an excess of a 5 per cent solution caused corneal necrosis.[3]

GENERAL METHODS EMPLOYED

Greenhouse plants were sprayed on a turntable either with a DeVilbiss spray gun (Type CH 6-2041) or a small bottle atomizer, according to the methods of McCallan and Wellman (15, 16). Spray applications on the small field plots (12×12 ft.) were made with a hand-operated 3-gallon Hudson CO_2 pressure sprayer. Applications on 0.1 acre plots were made with a tractor-drawn Kupfer power sprayer. This sprayer had a 20-foot boom with nozzle spacing of 9 inches. Sprays were applied with the boom about 12 inches above the ground. The sprays were generally applied at a pressure of 30 lb. per square inch, at the rates specified in each of the experiments.

In the laboratory tests, refined sodium 2,4-dichlorophenoxyethyl sulfate was generally used. In the field experiments production batches of approximately 90 per cent purity were employed. For comparative studies the sodium salt of 2,4-dichlorophenoxyacetic acid was used in the laboratory tests and the triethanolamine salt in field experiments.

LABORATORY RESULTS

RESPONSES WHEN APPLIED TO ABOVE-GROUND PORTIONS OF PLANTS

Applications of this chemical in aqueous solution or dust form to the foliage of such plants as tomato, induce little or no epinastic or formative effects. The results of a spray test comparing the effect of sodium 2,4-dichlorophenoxyethyl sulfate and the sodium salt of 2,4-D at concentrations of 10, 100, and 1,000 p.p.m. on potted tomato plants (*Lycopersicon esculentum* Mill. var. Bonny Best) are shown in Figure 1 A and B. It can be seen that 2,4-D was highly injurious at all concentrations, while the new herbicide was relatively non-injurious. About 8 days following spraying the new leaves showed some slight formative effects with the latter spray at 0.1 per cent. A similar test where the height of the plants was recorded after 11 days showed that 2,4-D at the same concentrations reduced growth to 4, 30, and 63 per cent of that of the controls, as compared to 93, 111, and 119 per cent for the new herbicide.

[3] Toxicological data from the Industrial Hygiene Fellowship, Mellon Institute, Pittsburgh, Pennsylvania.

FIGURE 1. Comparative responses of tomato plants to spray applications of sodium 2,4-dichlorophenoxyacetate (A) and sodium 2,4-dichlorophenoxyethyl sulfate (B), and to dust applications of both (C). A and B. 4 days after spraying. Left to right: control, 1,000, 100, and 10 p.p.m. C. Dusts at 1.0 per cent applied to single leaves. Left to right: control, Na 2,4-D, and sodium 2,4-dichlorophenoxyethyl sulfate, 6 days after treatment.

Since sodium 2,4-dichlorophenoxyethyl sulfate could be applied in dust form for weed control, it was of interest to determine whether dusts containing this chemical or 2,4-D would behave as differently as sprays. Either the entire tops or single leaves were dusted with preparations containing 1.0 or 0.1 per cent of the active ingredient in pyrophyllite. The soil surface was protected from contamination by using a paper disk. When single leaves were dusted they were enclosed in cellophane bags to prevent the dust from spreading to other parts of the plants. At both concentrations Na 2,4-D dusts produced marked epinastic responses, while no such effects were observed with sodium 2,4-dichlorophenoxyethyl sulfate dusts. The

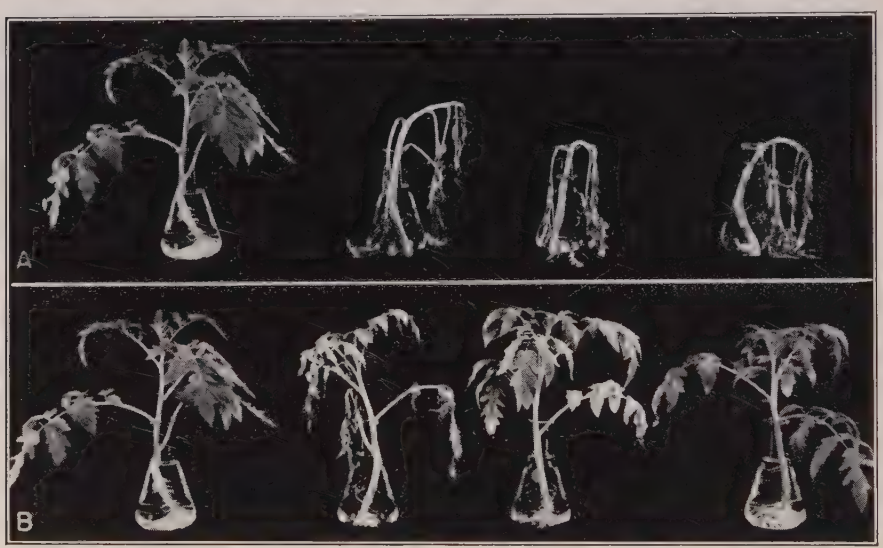


FIGURE 2. Responses of excised tomato plants eight days after immersion in solutions of Na 2,4-D (A), and sodium 2,4-dichlorophenoxyethyl sulfate (B). Left to right: control, 100, 10, and 1 p.p.m.

results of an experiment where single leaves were dusted appear in Figure 1 C.

The leaf immersion technique (11) was also used to compare the two compounds. A solution containing 500 p.p.m. of Na 2,4-D gave strong epinastic responses while a 1,000 p.p.m. solution of sodium 2,4-dichlorophenoxyethyl sulfate gave no such response in the same test. However, the latter compound caused brown necrotic areas in some of the leaflets in the top portions of the plant indicating that it had entered through the immersed leaflet.

The excised tomato plant technique of Zimmerman and Hitchcock (23) offered still another way to compare these two chemicals. Figure 2 shows such a comparison at concentrations of 1, 10, and 100 p.p.m. The highly

toxic effect of 2,4-D is evident even at 1 p.p.m. while the new herbicide produced only slight leaf injury at 100 p.p.m. and no injury at lower concentrations.

EFFECT OF SOIL ON HERBICIDAL PROPERTIES

When this new herbicide was tested in aqueous solutions in Petri dishes for effects on germination of radish (*Raphanus sativus* L. var. Red Globe), lettuce (*Lactuca sativa* L. var. Imperial 44), Italian rye grass (*Lolium multiflorum* Lam.), or sweet corn (*Zea mays* L.) little toxicant action was noted at 0.1 or 0.01 per cent. These results are in agreement with those given above. However, in tests in soil in the greenhouse this new herbicide was practically as toxic as Na 2,4-D against germinating seeds.

Using the cucumber root suppression test (19), a series of cultures was set up in which the effects of 100 p.p.m. of sodium 2,4-dichlorophenoxyethyl sulfate were studied with and without 5 grams of soil and with and without sterilization prior to planting of the cucumber seeds. Twenty-five seeds of cucumber (*Cucumis sativus* L. var. Davis' Perfect) were placed on filter paper in 6-inch Petri dishes to which 20 ml. of solution had been added. The cultures were maintained at 21° C. for five days after which root lengths were measured. Roots were greatly inhibited only in the presence of non-sterilized soil (Fig. 3), thus indicating that biotic action may be involved.

A series of culture dishes with soil (5 g. per dish) and sodium 2,4-dichlorophenoxyethyl sulfate at 100 p.p.m. were prepared and maintained at 21° C. After 0, 3, 6, 9, 24, and 48 hours sets of duplicates were removed and autoclaved immediately. Then 25 cucumber seeds were planted in each dish, as well as in a non-sterilized set. After five days the roots of each series were measured and the data for the time periods listed were 2.9, 0.7, 0.6, 0.5, 0.4, and 0.2 cm. respectively. It is evident that most of the action took place in the first three hours.

From the results of the above tests, an explanation may be found for the fact that when either sodium 2,4-dichlorophenoxyethyl sulfate or Na 2,4-D is applied in aqueous solutions to the soil surface of potted tomato plants strong epinastic responses are produced. In experiments in which the new herbicide and Na 2,4-D were added to the soil in a concentration series to single tomato plants (10), growth measurements and dry weights showed that, at concentrations necessary for weed control, both chemicals had markedly injured the highly sensitive tomato plants. The new herbicide was not as injurious as Na 2,4-D at the same concentrations; for example, at 0.5 lb. per acre the dry weight of Na 2,4-D treated tomatoes was approximately 50 per cent of untreated controls while the weight of tomato plants treated with 2,4-dichlorophenoxyethyl sulfate was 91 per cent of the controls. Height measurements in another experiment

gave similar results. Figure 4 shows the difference in response to the two chemicals. It is readily apparent that Na 2,4-D has considerably greater toxicity to the tomato plants than the new herbicide. The latter material, while producing epinastic responses when taken up by tomato plants from the soil, appears to be non-injurious to the growing point and recovery from formative effects generally follows from such treatments.

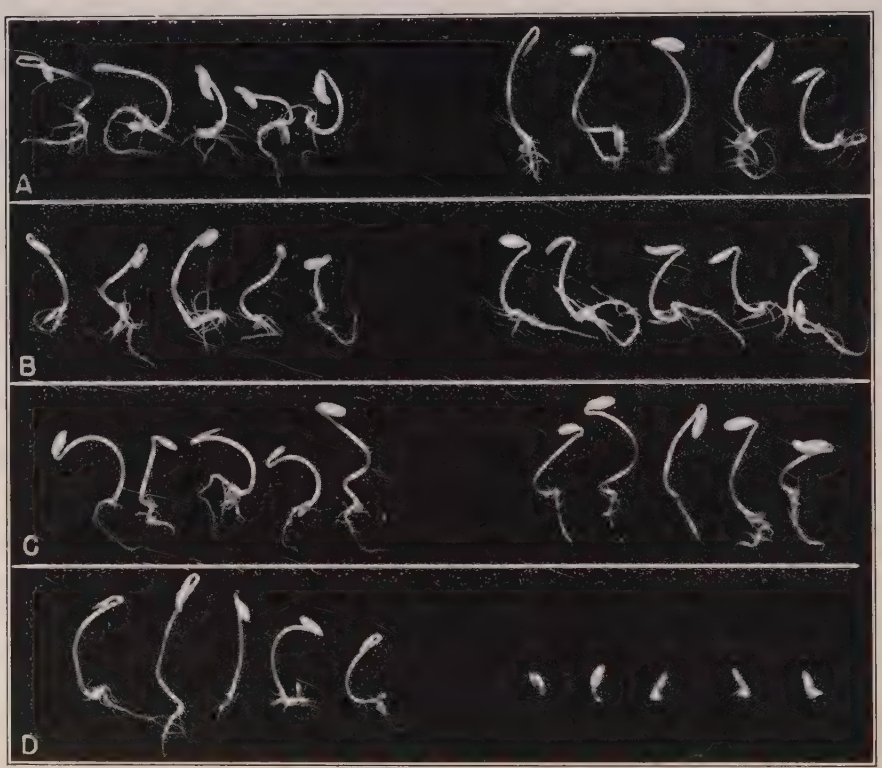


FIGURE 3. Effect of sterilization of soil on its ability to activate sodium 2,4-dichlorophenoxyethyl sulfate (100 p.p.m.) as shown by the retardation in growth of the roots of cucumber seedlings grown in Petri dishes. Left: control; right: sodium 2,4-dichlorophenoxyethyl sulfate. A. Sterilized series without soil. B. Non-sterilized series without soil. C. Sterilized soil series. D. Non-sterilized soil series.

EXPERIMENTS WITH GERMINATING SEEDS

In a test conducted in greenhouse seed flats (1.6 sq. ft. in area) in which a representative group of small seeds was planted, sodium 2,4-dichlorophenoxyethyl sulfate and Na 2,4-D were applied to the soil surface at rates of 1, 2, 4, 8, and 12 lb. per acre three hours after planting. The germination data recorded 18 days later are given in Table I. It is evident that in soil in the greenhouse these two chemicals are about equally toxic

TABLE I

GERMINATION OF FIVE SPECIES OF SEEDS* FOLLOWING APPLICATION OF SODIUM 2,4-DICHLOROPHENOXYETHYL SULFATE OR SODIUM 2,4-DICHLOROPHENOXYACETATE IN AQUEOUS SOLUTIONS TO THE SOIL SURFACE OF GREENHOUSE FLATS

Chemical	Lb. per acre	Percentage germination				
		Tomato	Beet	Onion	Spinach	Rye grass
Sodium 2,4-dichlorophenoxyethyl sulfate	1	86	17	70	23	96
	2	60	13	47	20	88
	4	30	10	20	23	36
	8	7	0	13	0	22
	12	23	0	7	0	28
Sodium 2,4-dichlorophenoxyacetate	1	100	3	47	13	92
	2	60	0	27	7	88
	4	30	3	33	17	32
	8	33	0	17	0	22
	12	7	0	7	0	4
Control	0	100	33	73	50	100

* Thirty seeds of tomato, beets, onions, and spinach, and 50 of rye grass were planted.

to small seeds. In a preliminary series of tests with large-seeded crops such as dent field corn (*Zea mays* L.), peas (*Pisum sativum* L.), and snap-beans (*Phaseolus vulgaris* L.) planted in greenhouse flats and treated three days following planting with the above two chemicals each at 5 lb. per acre, practically 100 per cent germination was obtained. Later field tests, however, have shown that sodium 2,4-dichlorophenoxyethyl sulfate had best be applied to such large-seeded crops as sweet corn as the seedlings are pushing through the soil, or to established plantings of such crops as asparagus or strawberries.

FIGURE 4. Responses of tomato plants 24 hours after the application of 50 ml. of herbicide solutions to the soil surface. Left to right: control, Na 2,4-D at 10,000 and 5,000 p.p.m., sodium 2,4-dichlorophenoxyethyl sulfate at 10,000 and 5,000 p.p.m.

RESIDUAL ACTION OF THE NEW HERBICIDE IN GREENHOUSE TESTS

The use of sodium 2,4-dichlorophenoxyethyl sulfate in the soil for weed control raised the question of just how long this chemical might per-

sist in an active form in the soil. A preliminary test was set up in which applications of this chemical at rates of 1 and 10 lb. per acre were made to the surface of soil in greenhouse flats. Eleven days after these applications lettuce seed was planted in the flats. Only 4 per cent germinated at the 1 lb. rate, and none germinated at the 10 lb. rate. Another planting of lettuce was made in the same flats 30 days following the initial chemical application. At this date germination averaged about 80 per cent which was practically the same as in the control planting. Thus, under the conditions of this test sodium 2,4-dichlorophenoxyethyl sulfate exhibited no toxic properties to germinating lettuce seeds 30 days after application. This would indicate that the chemical had been rendered inactive in the soil during this period of time.

TABLE II

RESIDUAL ACTIVITY OF 2,4-DICHLOROPHENOXYETHYL SULFATE AND SODIUM 2,4-DICHLOROPHENOXYACETATE AS DETERMINED BY THE GERMINATION OF TURNIP SEEDS (VAR. WHITE GLOBE) PLANTED AT VARIOUS INTERVALS FOLLOWING THE APPLICATION TO THE SOIL SURFACE OF GREENHOUSE FLATS

Chemical	Rate in lb. per acre	Percentage germination of turnip seeds planted on various days following treatment				
		0	3	6	9	12
Sodium 2,4-dichlorophenoxyethyl sulfate	1½	7*	41	48	60	77
	3	0	29*	54*	53*	88
Sodium 2,4-dichlorophenoxyacetate	1½	7*	39	48	47	81
	3	0	19*	48*	36*	85

* Seedling development retarded. Seed germination in the control was 100 per cent.

Another test involved the application of each chemical to the soil in small greenhouse flats at the rate of 1½ and 3 lb. per acre. Approximately 50 turnip (*Brassica rapa* L. var. White Egg) seeds, determined by weight, were sown in the flats the same day and at 3-day intervals over a 2-week period. The results appear in Table II. Practically all of the seeds that were planted on the day of treatment (0 days in the table) were killed by both chemicals, after 6 days at the 3 lb. rate about 75 per cent of the seeds were killed, after 9 days about 50 per cent of the seeds were killed, and after 12 days practically all of the seeds germinated. There thus appears to be no great difference between the rate of disappearance of the new herbicide and Na 2,4-D.

TESTS OF COMPOUNDS RELATED TO SODIUM 2,4-DICHLOROPHENOXYETHYL SULFATE

Data on the root suppression of cucumber seeds by a series of compounds related to sodium 2,4-dichlorophenoxyethyl sulfate are presented in Table III. The chemicals were used at a concentration of 10 p.p.m. in

the presence and in the absence of 5 grams of soil per dish. In aqueous solution at this concentration all of these compounds exhibited inactivity similar to that of the new herbicide. However, in the presence of soil the root growth was greatly suppressed. This would indicate that all are active in the presence of soil.

TABLE III

EFFECT OF COMPOUNDS (AT 10 P.P.M.) RELATED TO SODIUM 2,4-DICHLOROPHENOXYETHYL SULFATE ON THE ROOT GROWTH OF CUCUMBER SEEDS IN PETRI DISHES WITH AND WITHOUT SOIL

Compound	M.P., °C.	Without soil		With soil	
		Av. growth in cm.	Percentage of control	Av. growth in cm.	Percentage of control
Sodium 2,4-dichlorophenoxyethyl sulfate	190	3.05	96.2	0.41	30.5
Sodium 2,4,5-trichlorophenoxyethyl sulfate	242	2.27	71.6	0.50	37.3
Morpholine 2,4-dichlorophenoxyethyl sulfate	102	3.21	101.2	0.46	34.3
Ammonium 2,4-dichlorophenoxyethyl sulfate	273d	3.07	96.8	0.42	31.3
Calcium 2,4-dichlorophenoxyethyl sulfate	225d	2.73	86.1	0.46	34.3
Triethanolamine 2,4-dichlorophenoxyethyl sulfate	165	2.90	91.4	0.45	33.5
Sodium 4-chlorophenoxyethyl sulfate	225d	2.55	80.4	0.50	37.3
Control		3.17	100.0	1.34	100.0

FIELD RESULTS

Following the disclosure of the unique properties of sodium 2,4-dichlorophenoxyethyl sulfate, a large number of tests under a wide variety of field conditions have been conducted with this chemical. Sprays applied at many different times with respect to the appearance of the weeds or the crop have been investigated. The widely used term pre-emergence has not been employed in this paper. Pre-emergence treatments have been defined as the applications of chemicals to control weeds following planting but prior to crop emergence whether or not weeds have emerged (8). As such it is distinguishable from preplanting treatments. A further subdivision of pre-emergence has become necessary to distinguish between applications made before or after the germination of weeds. The term residual pre-emergence has been suggested (3) for the application prior to the

emergence of weeds. However, the term soil treatment seems preferable for this latter method, and is used in this paper. The status of pre-emergence weeding has been recently reviewed by Willard (22). Various forms of 2,4-D, particularly the amine salts, have been widely used in all of these methods of weed control (4, 8, 14). In addition, certain derivatives of dinitrophenol have been reported successful as soil treatment applications for weed control (3).

Stahler (21) in South Dakota has noted that sodium 2,4-dichlorophenoxyethyl sulfate in pre-emergence tests at 2 and 4 lb. per acre had some injurious effect on flax and soybeans but still indicated a need for further testing at other rates. He found that this chemical demonstrated a greater degree of crop species selectivity than any of a number of the newer herbicides included in the tests. Applications on an established stand of alfalfa gave good control of annual weeds and no injury to the alfalfa (6).

Since aqueous solutions of sodium 2,4-dichlorophenoxyethyl sulfate are practically non-toxic to plant foliage, sprays of this chemical may be used in established plantings for weed control. Tests in New Jersey by Gilbert and Wolf (7) on strawberry plantings have shown good weed control without injury to the strawberry plants. The application of 3 and 6 lb. per acre to a planting of the variety Sparkle in late spring gave excellent control of weeds for a period of six weeks when applied immediately following complete tillage. Summer applications of 3 and 6 lb. per acre (on August 17) to a planting of 11 strawberry varieties immediately following a thorough hoeing gave excellent control of weeds, particularly chickweed (*Stellaria media*) for six weeks with no apparent injury to the strawberry plants. It was not until eight weeks after the sprays had been applied that a new crop of chickweed seedlings began to appear. However, at this time the control plots were completely overgrown with chickweed. Further confirmatory studies have been made by Puffer and Aldrich (18). In strawberry plantings in Michigan Carlson and Moulton (5) have obtained good weed control without crop injury in tests with the new herbicide.

WEED CONTROL IN SWEET CORN

A series of tests were made on sweet corn plantings employing small plots (12×12 ft.) at the Institute farm in Yonkers and larger plots at the Seabrook Farms near Bridgeton, N. J. A replicated test was made in a field heavily infested with seeds of crab grass (*Digitaria* spp.) and purslane (*Portulaca oleracea* L.) with sodium 2,4-dichlorophenoxyethyl sulfate applied at the rate of 2 lb. per acre and the triethanolamine salt of 2,4-D at 1 lb. per acre. Applications were made three days and 14 days after planting. As shown by the data in Table IV sodium 2,4-dichlorophenoxyethyl sulfate gave better control of both broad-leaved weeds and grasses than did 2,4-D without injuring the corn or suppressing growth materially as measured by the green weight of tops 90 days after application.

In the tests at Seabrook Farms sodium 2,4-dichlorophenoxyethyl sulfate and the triethanolamine salt of 2,4-D were sprayed on plots of sweet corn (var. Golden Cross) at 0, 4, and 7 days after planting on July 28. The corn was emerging 4 days after planting, and at 7 days it was approximately four inches tall. Each plot contained six rows of corn, 200

TABLE IV

WEED CONTROL IN GOLDEN CROSS SWEET CORN SPRAYED THREE AND 14 DAYS AFTER PLANTING WITH SODIUM 2,4-DICHLOROPHENOXYETHYL SULFATE OR THE TRIETHANOLAMINE SALT OF 2,4-D*

Chemical	Rate in lb. per acre	No. weeds per sq. ft.		Injury to corn	Corn yield as green wt. of tops in lb. per plot
		Broad-leaved weeds	Grass weeds		
Sodium 2,4-dichlorophenoxyethyl sulfate	2	5.8	7.2	None	53.3
2,4-D Triethanolamine salt (40% acid)	1	25.3	54.4	Slight	54.0
Control	0	128.0	116.6	None	58.5

* Tops harvested from 30 feet of row in a single replicate 90 days after planting on July 5, 1949.

feet long. Water solutions of both materials were applied at the rate of $\frac{1}{2}$, 1, 2, and 3 lb. per acre in 40 gallons of water. Weed counts were taken 28 days after planting by counting the number of broad-leaved and grass weeds in a 6-inch square over 24 randomized sections of the plot. Results are expressed as number of weeds per sq. ft. and recorded in Table V and illustrated in Figure 5. It will be realized that a weed population as heavy as that shown in Figure 5 for the untreated control may offer, even after cultivation, serious competition to the corn at a later stage of development.

The best control of both broad-leaved and grass weed species was obtained when the applications were made four days after fitting. The broad-leaved weeds, principally lamb's quarters (*Chenopodium* sp.), were effectively controlled by both herbicides at all intervals. Crab grass (*Digitaria* spp.), however, was best controlled when incipient germination occurred over a period of several days before the herbicides were applied. Twenty per cent more crab grass was controlled when application of both herbicides was delayed until four days after planting. It was also noted that sodium 2,4-dichlorophenoxyethyl sulfate at 2 and 3 lb. per acre gave satisfactory control of crab grass when applied seven days after planting, whereas the amine salt of 2,4-D at $\frac{1}{2}$ and 1 lb. per acre of acid equivalent did not control crab grass effectively when applied at this time. An examination of the corn plants did not reveal any injury or physiological effects except in the plot treated with 2,4-D amine salt at 1 lb. per acre seven

FIGURE 5. Weed control in sweet corn (var. Golden Cross) following a 2 lb. per acre application of sodium 2,4-dichlorophenoxyethyl sulfate in plots at Seabrook Farms on July 28, 1950. Photographed August 24, 1950. A. Sodium 2,4-dichlorophenoxyethyl sulfate. B. Control. (*Photograph courtesy of Seabrook Farms.*)

days after planting. The corn in this plot showed the typical "onion leaf" rolling of the leaves.

TABLE V

EFFECT OF THE TIME OF SPRAY APPLICATIONS WITH SODIUM 2,4-DICHLOROPHENOXYETHYL SULFATE AND THE TRIETHANOLAMINE SALT OF 2,4-DICHLOROPHENOXYACETIC ACID ON WEED CONTROL IN SWEET CORN*

Chemical	Days after planting when applied	Rate in lb. per acre	Weed counts after 28 days	
			Av. No. per sq. ft.	
			Broad-leaved weeds	Grass weeds
Sodium 2,4-dichlorophenoxyethyl sulfate	0	1	3.7	2.5
		2	1.3	2.7
Triethanolamine salt of 2,4-dichlorophenoxyacetic acid	0	1	3.2	2.5
Sodium 2,4-dichlorophenoxyethyl sulfate	4	2	0.7	1.0
		3	0.3	1.0
Triethanolamine salt of 2,4-dichlorophenoxyacetic acid	4	1	1.2	1.5
Sodium 2,4-dichlorophenoxyethyl sulfate	7	2	0.8	1.0
		3	1.7	1.3
Triethanolamine salt of 2,4-dichlorophenoxyacetic acid	7	½	3.5	3.8
		1	3.8	2.8
Control		0	25.6	7.4

* Variety Golden Cross planted July 28, 1950, at Seabrook Farms, Bridgeton, New Jersey.

WEED CONTROL IN ASPARAGUS

Weed control experiments on an established asparagus (*Asparagus officinalis* L. var. *altilis* L.) planting were also conducted at Seabrook Farms. Sodium 2,4-dichlorophenoxyethyl sulfate and calcium 2,4-dichlorophenoxyethyl sulfate were applied four and three times respectively at approximately three-week intervals at the rate of 2 lb. per acre to two rows of asparagus each 570 feet long. The cultivator was run between the rows with its shovels adjusted so that soil would not be thrown up on the pair of rows where it would disturb the treated area. The control plot was also cultivated in the same manner and weeds developing in the row were not destroyed. The fourth and final application was made after the cutting season when the field had been disked and a fertilizer application made.

TABLE VI

WEED CONTROL AND YIELD DATA FROM ASPARAGUS FIELD PLOTS SPRAYED WITH SODIUM 2,4-DICHLOROPHENOXYETHYL SULFATE OR CALCIUM 2,4-DICHLOROPHENOXYETHYL SULFATE

Chemical	Rate in lb. per acre	Av. No. weeds per sq. ft.		Yield in lb. per row	
		Broad-leaved weeds	Grass weeds	Replicate 1	Replicate 2
Sodium 2,4-dichlorophenoxyethyl sulfate*	2	0.5	0.2	85.0	112.7
Sodium 2,4-dichlorophenoxyethyl sulfate**	2	0.4	0.2	108.2	93.0
Calcium 2,4-dichlorophenoxyethyl sulfate**	2	0.2	0.3	136.5	72.7
Control†	0	90.4	8.0	72.7	101.5

* Treated 5/2/50 in spray volume of 50 gal. per acre.
** Treated 4/27/50 in spray volume of 25 gal. per acre.
† The average weight of spears in a control plot that was hilled under standard cultivation practices was 89.8 lb.

The weed population was determined thirteen days after the first application by counting the number of broad-leaved and grass weeds within a 6-inch square over 48 randomized sections of the plot. The values expressed as the number of weeds per square foot are given in Table VI. Excellent weed control was maintained with both herbicides for three weeks following each treatment. The weed species controlled were velvetleaf (*Abutilon theophrasti* L.), lamb's quarters, and crab grass. Weed control in the mature asparagus following the final application is shown in Figure 6.

DISCUSSION

Sodium 2,4-dichlorophenoxyethyl sulfate is apparently the first organic chemical to be suggested for use as a practical herbicide, which does not have hormonal action when applied to foliage but does have such action through the roots after contact with the soil. The new herbicide has been effective in a variety of soils in many different parts of the United States and Hawaii.

The lack of plant response when applied to the foliage has important effects on the practical use of this chemical as a herbicide. The advantages in regard to lack of hazard of injury to adjoining crops by drift are apapparent; in the soil it exhibits its greatest effectiveness against germinating seeds. Of course, it is equally injurious to weed seeds and to crop seeds, especially so under conditions of high moisture. Applications are best made then at the time of crop seedling emergence. The reason that this chemical

FIGURE 6. Weed control in asparagus following applications of sodium 2,4-dichlorophenoxyethyl sulfate in plantings at Seabrook Farms. Photographed August 17, 1950. Both treated and control plots received the last cultivation on July 5, 1950. A. Sodium 2,4-dichlorophenoxyethyl sulfate applied on July 8 at 2 lb. per acre making a total application of 8 lb. per acre for the season. B. Control. (*Photograph courtesy of Seabrook Farms.*)

can be used to control germinating weed seeds without injury to sweet corn seedlings or to an established planting of strawberries or asparagus, is that the herbicide does not affect plants from foliage applications. Since it is applied to the surface of the soil, it is most abundant in the upper layer and consequently the deeper root systems of the corn seedlings or strawberry or asparagus plants absorb little, if any, of the herbicide. As the experiments have shown, the toxicity of the new herbicide to roots or to the growing regions is considerably less than that of 2,4-D. Thus when applications are made to snapbeans or lima beans as they emerge, the foliage is rarely injured, but later, as the trifoliate leaves appear, some formative effects may be observed. These soon disappear since the growing zones are not permanently injured. Under such conditions, sprays with 2,4-D would be extremely injurious.

Similarly, injury or lack of injury to a crop or weed may be determined by whether or not shallow roots are present to absorb a toxic amount of the effective material from the upper layer of the soil. In a number of tests in which applications were made 6 to 10 days following tillage when small weed seedlings less than one-half inch high were present, enough of the material was apparently absorbed by the small roots to dwarf these seedlings and thus give effective weed control.

The finding of the unusual properties of this chemical has already led other investigators to establish its usefulness in weed control in strawberries, alfalfa, potatoes, sugar cane, and gladiolus. Tomatoes and cotton have been found to be sensitive to soil applications of this new herbicide but tomatoes, at least, are not injured by application to the foliage only. Crops of intermediate sensitivity to soil applications are snapbeans (12) and lima beans (13). Extensive tests on Ben Fish baby lima beans at Seabrook Farms on sandy loam soils have shown that in the early spring plantings of April and May injury may occur from spray applications made too soon following planting. Not only is this due to the higher soil moisture levels prevailing during this period, but also to the fact that the germination of the beans is not sufficiently uniform to apply the sprays at total emergence. With increasing soil temperature in June, the germination is more uniform and sprays applied just after total emergence have given good weed control without crop injury.

SUMMARY

In tests with a large number of compounds, sodium 2,4-dichlorophenoxyethyl sulfate was found to be relatively non-toxic when applied directly in aqueous solutions of low concentration to the foliage of plants or to germinating seeds but to be highly herbicidal when used in combination with soil. Sterilization experiments showed that the effectiveness is largely the result of biotic action. Lack of injury from spray or dust applications

to foliage is in marked contrast to such applications from 2,4-D. Danger of injury to adjoining crops from drift, therefore, is not a factor when sodium 2,4-dichlorophenoxyethyl sulfate is used.

Studies of the rate of disappearance of the new herbicide in soil under greenhouse conditions have indicated that the time required ranges from 12 to 30 days when 1 to 10 lb. per acre is applied.

In large scale tests on sweet corn (var. Golden Cross) the best weed control was obtained when sodium 2,4-dichlorophenoxyethyl sulfate was applied several days after the germination of weed seeds had begun. Good control of broad-leaved weeds was obtained with a 2 or 3 lb. per acre application when used the day of planting, or four or seven days later. Best control of crab grass was obtained when the application was made four days after fitting the soil. Good weed control was obtained in an established planting of asparagus when sodium or calcium 2,4-dichlorophenoxyethyl sulfate was applied at 2 lb. per acre at three intervals during the cutting season, and again at the end of the cutting season, and without the customary practice of hilling during cultivation. Asparagus yields and quality were not affected by these treatments.

These results thus show that sodium 2,4-dichlorophenoxyethyl sulfate is a valuable addition to the chemicals now available for use as herbicides.

LITERATURE CITED

1. AKAMINE, ERNEST K. Plant-growth regulators as selective herbicides. Hawaii Agric. Exp. Sta. Circ. 26. 43 pp. 1948.
2. ALLARD, R. W., H. R. DEROSE, and C. P. SWANSON. Some effects of plant growth-regulators on seed germination and seedling development. Bot. Gaz. **107**: 575–583. 1946.
3. BARRONS, KEITH C. The phenolic herbicides. Proc. Northeastern States Weed Control Conf. New York City, Jan. 1949. Pp. 219–224.
4. CAREW, JOHN. Chemical weeding of vegetables. New York [Ithaca] State Coll. Agric. Cornell Ext. Bull. 769. 16 pp. 1949.
5. CARLSON, ROBERT F., and J. E. MOULTON. Further testing of herbicides in strawberry plantings. Proc. Northeastern States Weed Control Conf. New York City, Jan. 1951. [In press].
6. DUTTON, W. C. Results with newer herbicides in 1949. Proc. North Central Weed Control Conf. 6th ann. meeting, Sioux Falls, So. Dak., Dec. 1949. Pp. 20–22.
7. GILBERT, F. A., and D. E. WOLF. Effects of some herbicides on strawberry plants of various varieties. Proc. Northeastern States Weed Control Conf. New York City, Jan. 1950. Pp. 127–129.
8. GRIGSBY, B. H., BOYD R. CHURCHILL, CHARLES L. HAMNER, and ROBERT F. CARLSON. Chemical weed control. Michigan Agric. Exp. Sta. Circ. 214. (Rev. ed.) 38 pp. 1950.
9. HAMNER, C. L., J. E. MOULTON, and H. B. TUKEY. Effect of treating soil and seeds with 2,4-dichlorophenoxyacetic acid on germination and development of seedlings. Bot. Gaz. **107**: 352–361. 1946.
10. HITCHCOCK, A. E., and P. W. ZIMMERMAN. Absorption and movement of synthetic growth substances from soil as indicated by the responses of aerial parts. Contrib. Boyce Thompson Inst. **7**: 447–476. 1935.

11. KING, LAWRENCE J. A leaf immersion technique for studying the absorption and translocation of chemicals in plants. Contrib. Boyce Thompson Inst. **15**: 165–171. 1948.
12. ———. Dichloral urea (Experimental Herbicide 2) and sodium 2,4-dichlorophenoxyethyl sulfate (Experimental Herbicide 1) as selective herbicides. Proc. Northeastern States Weed Control Conf. New York City, Jan. 1950. Pp. 302–309.
13. KING, L. J., and J. A. LAMBRECH. A new chemical for use in pre-emergence weeding. Proc. Northeastern States Weed Control Conf. New York City, Jan. 1949. Pp. 34–36.
14. LACHMAN, WILLIAM H. Weed control in vegetable crops. Massachusetts Agric. Exp. Sta. Bull. 451. 60 pp. 1948.
15. MCCALLAN, S. E. A. Some improvements in equipment for evaluating fungicides by the foliage disease method. Contrib. Boyce Thompson Inst. **15**: 71–75. 1948.
16. MCCALLAN, S. E. A., and R. H. WELLMAN. A greenhouse method of evaluating fungicides by means of tomato foliage diseases. Contrib. Boyce Thompson Inst. **13**: 93–134. 1943.
17. MITCHELL, JOHN W., and PAUL C. MARTH. Germination of seeds in soil containing 2,4-dichlorophenoxyacetic acid. Bot. Gaz. **107**: 408–416. 1946.
18. PUFFER, R. E., and R. J. ALDRICH. Two years results on the use of certain herbicides for weed control in different varieties of strawberries. Proc. Northeastern States Weed Control Conf. New York City, Jan. 1951. [In press].
19. READY, DANIEL, and VIRGINIA GRANT. A rapid sensitive method for determination of low concentrations of 2,4-dichlorophenoxyacetic acid in aqueous solution. Bot. Gaz. **109**: 39–44. 1947.
20. SLADE, R. E., W. G. TEMPLEMAN, and W. A. SEXTON. Plant-growth substances as selective weed killers. Differential effect of plant-growth substances on plant species. Nature [London] **155**: 497–498. 1945.
21. STAHLER, L. M. Screening of new selective herbicides. Proc. North Central Weed Control Conf. 6th ann. meeting, Sioux Falls, So. Dak., Dec. 1949. Pp. 95–101.
22. WILLARD, C. J. Pre-emergence control of weeds in field crops. Proc. North Central Weed Control Conf. 6th ann. meeting, Sioux Falls, So. Dak., Dec. 1949. Pp. 19–20.
23. ZIMMERMAN, P. W., and A. E. HITCHCOCK. Tropic responses of leafy plants induced by application of growth substances. Contrib. Boyce Thompson Inst. **9**: 299–328. 1938.
24. ———. Substituted phenoxy and benzoic acid growth substances and the relation of structure to physiological activity. Contrib. Boyce Thompson Inst. **12**: 321–343. 1942.

GROWTH-REGULATING EFFECTS OF CHLOROSUBSTITUTED DERIVATIVES OF BENZOIC ACID[1]

P. W. Zimmerman and A. E. Hitchcock

Physiological activity of substituted derivatives of benzoic acid has been repeatedly mentioned in publications with special reference to formative influences, including flowering habit, modification of leaves, and correlation of organs (7, 8, 9, 10). Until the work of Bentley (1), however, not enough experimental data had been provided to support the claims that such compounds had cell elongating activity. Using the straight growth method with *Avena* coleoptiles, Bentley reported high physiological activity for 2,3,6-trichlorobenzoic acid and 2,3,6-trichlorobenzaldehyde. It was further shown that 2,3,6-trichlorobenzaldehyde was more active than β-indoleacetic acid or 2,4-dichlorophenoxyacetic acid when used at the rate of 1 mg. per liter of solution applied to coleoptile sections.

These results are of considerable interest since the new chemicals do not fulfill the structural requirements of Koepfli *et al.* (2), Veldstra (4, 5), or Muir (3) for physiological activity. Koepfli *et al.* stated that to be physiologically active the molecule must have an unsaturated ring system and a side chain carrying a carboxyl group at least one carbon atom removed from the ring. The hypotheses of Veldstra call for a basal ring system with high surface activity and a carboxyl group in a very definite spatial position with respect to the ring system. Muir postulated further that at least for the phenoxy derivatives of the lower fatty acids the position on the benzene ring adjacent to the point of attachment of the side chain is directly involved in growth reactions and that if a compound is to have activity at least one of the ortho positions needs to be free. The 2,3,6-trichlorobenzoic acid and the corresponding aldehyde do not meet these requirements.

2,3,6-Trichlorobenzoic acid and the aldehyde derivative, supplied by Dr. W. A. Sexton, Imperial Chemical Industries, Ltd., England (the same source of supply as that used by Bentley), were tested in this laboratory and compared with the activity of several other chemicals. The purpose of this report is to show that these two compounds induce cell elongation as reported by Bentley and also cause responses in dicotyledonous plants similar to those of substituted phenoxy, indole, and naphthalene compounds.

Tomato (*Lycopersicon esculentum* Mill.), Turkish tobacco (*Nicotiana*

[1] This article was preprinted Feb. 16, 1951.

Copyright, 1951, by Boyce Thompson Institute for Plant Research, Inc.

tabacum L.), and stevia (*Piqueria trinervia* Cav.) plants were used as test objects. Solutions of the 2,3,6-trichlorobenzoic acid and the corresponding aldehyde were applied to the aerial parts of plants and to soil in which the plants were growing. Lanolin preparations were applied to various portions of the aerial parts of test objects.

The responses induced were similar regardless of the method of treatment. One per cent water solutions of both the acid and the aldehyde were lethal when sprayed on leaves, the plants dying within six days after the treatment. When applied to soil at the rate of 1 mg. per 100 cc. of soil both chemicals caused pronounced responses of tomato plants.

Lanolin preparations were used at a concentration range of 0.01 to 100 mg. per gram. A dose ratio of 2 to 2.5 was used in the principal concentration series. When applied at 100 mg. per gram of lanolin to the stem of a plant the chemicals were lethal. At 1 to 50 mg. per gram both the acid and the aldehyde caused curvatures, proliferations, induction of roots, and later modification of leaves and other organs which developed after the treatment. Table I shows the threshold concentrations required to induce the various responses. Figure 1 illustrates some of the responses induced with 2,3,6-trichlorobenzoic acid.

TABLE I

THE THRESHOLD VALUES (MG. OF CHEMICAL PER GRAM OF LANOLIN) WHICH BARELY INDUCED RESPONSES WHEN 2,3,6-TRICHLOROBENZOIC ACID AND THE CORRESPONDING ALDEHYDE WERE APPLIED TO TOMATO PLANTS

Responses induced	Acid	Aldehyde
Modification of leaves	0.01	0.025
Cell elongation (curvatures)	0.25	0.25
Induction of roots	5.0	10.0
Inhibition of growth	50.0	50.0
Lethal	100.0	100.0

When used at a concentration of 10 mg. per gram of lanolin, the time required for the acid and the aldehyde to bring about a measurable curvature response was approximately one hour. In two hours the angle between the treated leaf and the stem increased from 60° to 105°. In the same time the aldehyde caused an increase from 62.5° to 77.5°. Within three hours the response tended to become systemic and in 24 hours all of the leaves moved downward, nearly paralleling the stem. At this stage of response the plants resembled those treated with α-naphthaleneacetic, 2,4-dichlorophenoxyacetic acids, or other hormone-like substances. The initial response was slightly slower than that of α-naphthaleneacetic acid but occurred at approximately the same rate as that induced with phenylacetic acid. It appears, therefore, that these two substances act directly to cause cell elongation rather than on precursors which may become active ma-

terial. If this supposition be true, then some derivatives of benzoic acid may be classified as hormone-like substances from another point of view. They have already been so classified from their capacity to induce formative effects and parthenocarpic development of fruit (7, 8, 9, 10).

FIGURE 1. The response of tomato plants to treatment with lanolin preparations of 2,3,6-trichlorobenzoic acid and the corresponding aldehyde. A. Left to right: control; plants 29 days after treatment with preparations of 50, 25, and 0.5 mg./gm. lanolin. B. Left to right: control; modified leaves and fruit which grow after treatment with preparations containing 10 mg. of the acid (middle) and the aldehyde.

Formative effects are induced with much lower concentrations of the chemicals than required for other responses. Table I shows that for the acid the threshold value for modification of leaves is 0.01 mg. per gram of lanolin applied to the stem of the plant. Figure 1 A shows one plant having pronounced modification of leaves induced with 1 mg. per gram.

Fruit were modified with concentrations of 1 to 25 mg. per gram (Fig. 1 B).

Parthenocarpy was induced when water solutions containing 50 mg. of the acid per liter were sprayed on the flower clusters. Threshold values were not determined for this response. Also seedless fruit was produced by tomato plants treated with lanolin preparations. In this case flower buds appeared after the chemical was applied. Both calyx and ovaries were modified in shape from treatment with a lanolin preparation containing 10 mg. of the acid per gram of solution (Fig. 1 B). As the plants continued to grow the chemical effects were lost and normal leaves and flowers appeared.

2,3,6-Trichlorobenzoic acid was translocated by stevia stems more readily than naphthaleneacetic acid or indolebutyric acid. The latter chemicals induced roots locally when applied to the stem without signs of translocation. The derivatives of benzoic acid, however, appeared to move readily to the tip where they induced typical modification of leaves and knob-like swellings of the stem. They moved downward a distance of five nodes and then upward on axillary branches. The upward movement was more pronounced than downward movement when the chemical was applied around the stem.

The capacity of plants to translocate hormone-like material appears to vary with the species as well as the substance involved. For example, naphthaleneacetic acid is readily translocated by tomato but not by stevia plants. The latter, however, translocates 2,3,6-trichlorobenzoic acid readily but not indolebutyric acid. Many other variations could be listed and this fact shows that, if results of different laboratories are to be compared, standard methods and test objects must be used.

Since the new derivatives of benzoic acid cause cell elongation and proliferation, induce adventitious roots, and modify leaves, they can be added to the well known list of hormone-like substances. This admission, however, does not simplify the problem of finding what makes a chemical active or how it functions in the plant. As previously stated, growth is a complex process, depending upon many simultaneous, closely integrated reactions involving a large number of different substances (6, p. 340; 8, p. 341). It is interesting to find that the trend of growth can be influenced by simply providing a foreign substance, like a substituted benzoic acid, to work with the naturally occurring complex mechanism in plants.

SUMMARY

2,3,6-Trichlorobenzoic acid and 2,3,6-trichlorobenzaldehyde caused cell elongation and proliferation of tissue, induced adventitious roots, modified the pattern of leaves and other organs, and caused parthenocarpic development of fruit. It was concluded, therefore, that these chemicals

should be included with the list of well known hormone-like substances for plants.

LITERATURE CITED

1. BENTLEY, JOYCE A. Growth-regulating effect of certain organic compounds. Nature [London] **165**: 449. 1950.
2. KOEPFLI, J. B., KENNETH V. THIMANN, and F. W. WENT. Phytohormones: Structure and physiological activity. I. Jour. Biol. Chem. **122**: 763–780. 1937–1938.
3. MUIR, R. M., C. H. HANSCH, and A. H. GALLUP. Growth regulation by organic compounds. Plant Physiol. **24**: 359–366. 1949.
4. VELDSTRA, H. Researches on plant-growth substances. IV. Relation between chemical structure and physiological activity. I. Enzymologia **11**: 97–136. 1944.
5. ———. Researches on plant growth substances. V. Relation between chemical structure and physiological activity. II. Contemplations on place and mechanism of the action of the growth substances. Enzymologia **11**: 137–163. 1944.
6. ZIMMERMAN, P. W. Responses of plants to hormone-like substances. Ohio Jour. Sci. **37**: 333–348. 1937.
7. ———. The formative influences and comparative effectiveness of various plant hormone-like compounds. Torreya **43**: 98–115. 1943.
8. ZIMMERMAN, P. W., and A. E. HITCHCOCK. Substituted phenoxy and benzoic acid growth substances and the relation of structure to physiological activity. Contrib. Boyce Thompson Inst. **12**: 321–343. 1942.
9. ———. Flowering habit and correlation of organs modified by triiodobenzoic acid. Contrib. Boyce Thompson Inst. **12**: 491–496. 1942.
10. ———. Formative effects of several substituted phenoxy acids applied to *Kalanchoe*. Contrib. Boyce Thompson Inst. **15**: 421–427. 1949.

GERMINATION OF SEEDS OF EICHHORNIA CRASSIPES SOLMS.

Lela V. Barton and Janet E. Hotchkiss

It has been known for a long time that the principal means of spread of *Eichhornia crassipes*, water hyacinth, is by vegetative propagation. However, many seeds are produced each year and enough seedlings may become established to pose serious threat of reinfestation following successful control of the vegetative parts of the plants by chemicals. This has been recognized by Hitchcock *et al.* (2, 3) who have published observations on the germination behavior of the seeds. From greenhouse tests and from an examination of the natural conditions under which seedlings were found, these authors concluded that the principal requirement for germination was a minimum water temperature of 28° to 30° C. Other authors (Haigh, 1; Penfound and Earle, 4) have had variable results with the germination of these seeds with and without sunlight. It is evident that the requirements of water hyacinth seeds for germination have not been definitely established. The present tests were conducted at the suggestion of Hitchcock and co-workers at this Institute, who have been concerned with the growth, reproduction, and the practical control of this species.

MATERIALS AND METHODS

Seeds were received in 1949 from New Orleans, Louisiana, through the courtesy of Henry Kirkpatrick, Jr., in two shipments. The shipments were collected and mailed on June 16 and June 29 and received on June 21 and July 5, respectively. Both lots were shipped in the capsules which were still attached to the flower stalks. Some of the darker-colored capsules fell off the stalks in transit, but they all arrived moist and in good condition. Seeds of the two shipments were removed from the capsules and thoroughly mixed for germination and storage tests. They were kept in water at 10° C. from the time they were received until the tests were begun (9 to 23 days), when they were divided into three lots, according to color, as follows: A, dark brown seeds, apparently full size; B, light brown seeds, apparently full size; C, dark cream or very light tan seeds, somewhat smaller than A and B. For convenience all of these will be referred to as fresh seeds.

Germination tests were set up on July 14. Four wide-mouthed bottles, each with one inch of water (about 20 ml.) and 100 seeds, were placed at each of the following air temperatures: greenhouse, constant temperatures of 10°, 15°, 20°, 25°, 30°, 35°, and 40° C. and daily alternations of 5° to

Copyright, 1951, by Boyce Thompson Institute for Plant Research, Inc.

30°, 5° to 35°, 5° to 40°, 5° to greenhouse, 15° to 30°, 15° to 35°, 15° to 40° and 15° to greenhouse. In the case of each alternating temperature, the cultures were transferred to the lower temperature for sixteen hours (at night) and the higher temperature for eight hours each day, five times weekly. They were left at the lower temperatures over the week ends. Also ten bottles with 100 seeds each in water were placed at an approximate temperature of −4° C., constant temperatures of 1°, 5°, 10°, 15°, 20°, 25°, 30°, 35°, and 40° C. and daily alternations of 5° to 30°, and 5° to 40° C. for four days, one week, two weeks, one month, and two months, at which times duplicate bottles were transferred to the greenhouse for germination. Cultures were kept in dark incubators at controlled temperatures but were exposed to diffuse light in the laboratory during transfers and examinations.

Some of the seeds of lots A, B, and C were allowed to dry in the laboratory for six days before germination tests were started. In addition, uncounted lots of seeds were stored in water at approximately −4°, and at 5°, 20°, 30°, and 40° C. Viability tests were made after four days, one and two weeks, and one, two, six, twelve and seventeen months under these conditions. Greenhouse temperatures reported were air temperatures except with the seeds stored for 17 months when water temperatures were taken.

RESULTS AND DISCUSSION

Germination. There was no germination up to fourteen weeks of either moist or dry fresh seeds kept at constant temperatures of 10° through 40° C. or at alternations of 15° to 30°, 15° to 35°, or 15° to 40° C. Some seedlings were produced at alternations of 5° to 30° and 5° to 35° C., and at 5° to 40° C. lot A gave 62 per cent germination, lot B, 48 per cent, and lot C, 15 per cent (Table I). Conditions in the greenhouse favored germination. Cultures of A, B, and C kept constantly in the greenhouse gave 95, 98, and

TABLE I

GERMINATION OF SEEDS IN WATER AT VARIOUS AIR TEMPERATURES

Germination temperature, °C.*	Per cent germination of different seed lots					
	Fresh seeds			Seeds stored at 20° C. for 17 mos.		
	A	B	C	A	B	C
5 to 30	11	3	23	96	97	0
5 to 35	9	3	5	—	—	—
5 to 40	62	48	15	97	97	2
5 to G.H.	90	90	25	93	97	8
15 to 30	0	0	0	1	1	0
15 to 40	0	0	0	78	70	0
15 to G.H.	89	85	41	78	90	6
G.H.	95	98	46	96	91	0

* G.H. = greenhouse.

46 per cent germination respectively. Air temperatures in the greenhouse during this period ranged from 23° to 59° C. The same seed lots gave 89, 85, and 41 per cent germination at a daily alternation of 15° C. to greenhouse and 90, 90, and 25 per cent at 5° C. to greenhouse.

Although the seeds germinated best at the high temperatures of the greenhouse, they seemed to do almost as well when placed at 5° or 15° C. for the night. Not only did water hyacinth seeds tolerate temperatures as low as 5° C. for sixteen-hour periods daily without subsequent impairment of germination but alternating cool and warm temperatures brought about germination when constant temperatures in a range which would be expected to favor germination, i.e. 25° to 40° C., failed.

Samples of the same seed lots were tested at various temperatures after 17 months of storage at 20° or 30° C. The behavior after storage at 20° C. is shown in Table I. It will be noted that these seeds which had become thoroughly after-ripened during their storage germinated over a wider temperature range and with percentages up to 97 at daily alternating temperatures of 5° to 30° C. and 5° to 40° C. without any direct sunlight. The apparent requirement of sunlight for complete germination of seeds not more than 28 days old from harvest and the loss of this requirement with increased length of the storage period is in agreement with the behavior of other light-favored seeds, such as lettuce for example.

It is significant that constant temperatures in a range which would be expected to favor germination failed both with fresh seeds and with those which had been stored in water at 20° C. for 17 months. The only seedlings secured at constant temperatures in the present tests were from seeds stored 17 months at 30° C. and germinated at 40° C. where 23 per cent of lot A seeds sprouted. Although water temperatures were not measured, the greenhouse air temperatures for the fresh seeds, germinated in July and August 1949, ranged from 23° to 59° C. On the other hand, the test with seeds stored for 17 months was made in December 1950 in a greenhouse with a thermostatic control set at 20° C., but where the water culture temperatures ranged from 21.5° to 32° C., with an average 9 A.M. temperature of 22.4° C. and an average 3 P.M. temperature of 26° C. daily for the first seven days during which time most of the germination took place. These results indicate an effect of light over and above that of increased temperature since these recorded temperatures are definitely below those required for germination without light. This was further indicated by germination up to 16 per cent in a greenhouse at temperatures of 8° to 24° C. with averages of 17.9° C. at 9 A.M. and 21.6° C. at 3 P.M. However, no germination took place within four weeks when the greenhouse temperature was reduced to a range of 5° to 19.5° C.

From the data at hand, then, it would seem that sunlight plays a definite role in the germination of dormant seeds or of non-dormant seeds held at unfavorable low temperatures. It is doubtful, however, that exposure to

TABLE II

EFFECT OF STORAGE IN WATER ON SUBSEQUENT GERMINATION PERCENTAGES. DUPLICATES OF 100 SEEDS EACH.

Seed		Storage time	Germination in water in greenhouse after storage at °C.											
Lot	Moist or dried		Approx. −4	1	5	10	15	20	25	30	35	40	5 to 30	5 to 40
A	Moist	4 days	97	88	99	96	100	99	85	99	99	98	98	87
		1 wk.	83	99	95	92	96	79	95	99	98	92	86	99
		2 wks.	12	3	5	15	42	65	73	41	96	96*	1	92
		1 mo.	62	94	97	97	96	98	99	97	91	95	92	97
		2 mos.	0	1	14	3	2	24	39	95	95	96	50*	—
		6 mos.	0	—	40	—	—	90	—	88	—	9	—	—
		12 mos.	—	—	15	—	—	33	—	97	—	3	—	—
		17 mos.	—	—	—	—	—	96	—	83	—	—	—	—
	Dried 6 days	2 wks.	95	0	1	0	0	0	5	0	0	1	2	11
		1 mo.	73*	86	0	0	98	0	0	0	37*	75	0	36
		2 mos.	4	25*	15	35	70	88	95	79	97	94	3	—
B	Moist	4 days	95	84	84	94	81	86	93	91	95	100*	94	95
		1 wk.	85	33	83	59*	86	61	87	90	95	96	96	95
		2 wks.	7	2	1	0	13	63*	60*	6	73	95	46*	72*
		1 mo.	27	84	87	86	86	91	77	88	83	95	92	93
		2 mos.	2	6	16	11	23	57	77	90	94	90	10	—
		6 mos.	1	—	93	—	—	93	—	95	—	1	—	—
		12 mos.	—	—	24	—	—	41	—	91	—	1	—	—
		17 mos.	—	—	—	—	—	91	—	85	—	—	—	—
	Dried 6 days	2 wks.	86	0	2	0	0	0	0	0	0	0	0	9
		1 mo.	56	2	1	0	85	77*	0	20*	1	95*	0	—
		2 mos.	10	20	38	77	84	91	88	95	99	69*	12	—
C	Moist	4 days	34*	0	0	0	0	0	0	0	0	0	—	0
		1 wk.	2	0	0	0	0	0	0	0	0	0	—	0
		2 wks.	0	0	0	0	0	0	0	0	0	0	—	3
		1 mo.	5	41	98*	19	0	40	45	64	59	59*	59*	3
		2 mos.	2	9	42	33	53	64	68	67	56	41	65	—
		6 mos.	0	—	33	—	—	43	—	47	—	1	—	—
		12 mos.	—	—	1	—	—	14	—	69	—	0	—	—
		17 mos.	—	—	—	—	—	0	—	23	—	0	—	—
	Dried 6 days	2 wks.	24	0	0	0	—	0	—	—	—	1	0	—
		1 mo.	13*	0	0	0	—	0	—	—	—	18*	0	—
		2 mos.	1	16	21	37	—	51	—	—	—	24	32	—

* Single culture.
— No test made.

diffuse light in the laboratory has any effect on germination. Hitchcock *et al.* have kept hyacinth seeds in water in the laboratory for more than two years without germination. Exact light requirements, i.e. intensity, wave length and time of exposure, effective for promoting germination, have not been determined.

Storage. This phase of the investigation yielded data on the dormancy of the seeds as well as their longevity under certain storage conditions. The storage conditions and the results obtained are shown in Table II.

Storage of seeds held moist from harvest and of those which had been dried for six days in the laboratory was made in one inch of water in bottles. Storage was in single large lots at each temperature. Duplicate samples of 100 seeds each were removed and placed in water in smaller bottles in the greenhouse for germination after storage for four days to 17 months. Since the experiment was started in July, transfers to the greenhouse were made in July, August, September, January, and December. This means that there was much variation in the greenhouse germination temperature for the different tests. In spite of this fact, however, there were consistent trends in germination response with the exception of the transfer made after two weeks of storage. This may have been caused by the excessively high greenhouse temperature at the time of transfer.

Some of the moist seeds of lots A and B were able to survive one month in ice at approximately $-4°$ C. and as long as two months at temperatures up to 15° C. At 40° C. also, the seeds not only survived for two months, but gave excellent germination after such storage. When the storage period was lengthened to six or twelve months, however, 20° and 30° C. proved better than 5° or 40° C. for keeping the seeds viable. This is rather surprising in view of the general beneficial effects of low temperature on the keeping quality of seeds. It should be kept in mind that these seeds were stored in water, whereas the usual storage condition for seeds of other species is dry. *Eichhornia* seeds which had been dried prior to storage in water responded in a similar manner as far as keeping quality was concerned (Table II).

It has been shown by Haigh (1) that *Eichhornia* seeds remain viable in water in the laboratory for at least five years, but that dry seeds fail to germinate after three years of storage. The life span of the seeds under the conditions of the present tests has not been determined.

In the matter of dormancy, the color and hence the possible maturity of the seed at harvest, as well as subsequent drying before storage, seemed to have an effect. For example, moist seeds of lot C required a longer period of storage than the darker-colored lots A and B before germination would proceed upon transfer to the greenhouse. Only a few seedlings were produced after four days or one or two weeks of storage, but extension of the storage time to one or two months allowed the seeds to after-ripen so that germination could proceed. Again, as in lots A and B, lot C seeds began to lose their viability after two months at low temperatures and at the very high temperature of 40° C., the best of the temperatures tried being 20°, 30°, and 35° C. Further tests were not made on dried seeds since the supply was exhausted after two months of storage.

Drying the seeds for six days before storing in water increased the dormancy of lots A and B, as can be seen in Table II.

The figures shown in this table are final germination percentages obtained regardless of the length of time required. The speed of germination

also is affected by storage. After only four days or one week of storage, the beginning of germination was delayed for three weeks and required six to eight weeks for completion. Storage for one month prior to placing in the greenhouse hastened the germination process so that a considerable number of seedlings had appeared in two weeks with a maximum of four weeks needed for full germination. Extension of the storage time to two months resulted in complete germination within one week after transfer from 30° C. to the greenhouse. It has been reported previously that water hyacinth seeds need a rest period of about two months in water to break the dormancy present at harvest (2, 3). The speed of germination as well as the final percentage is an index of the dormancy of any particular seed lot.

SUMMARY

From the data presented, it appears that a combination of high temperature and light is needed for complete germination of dormant seeds of *Eichhornia crassipes*. However, periods of eight hours a day at a temperature as low as 5° C. did not impair germination in the greenhouse, and daily alternating temperatures of 5° to 30°, 5° to 35°, and 5° to 40° C. permitted some germination in dark incubators. Constant temperatures of 30°, 35°, and 40° C. failed to bring about germination in the dark. After 17 months of storage in water at 20° or 30° C., the seeds became less dormant as evidenced by their germination over a wider range of temperatures, but alternating low and high temperatures or greenhouse conditions were still best. These seeds germinated at fluctuating air or water temperatures as low as 22° C. in the sunlight in the greenhouse. Thus sunlight favored the germination of dormant seeds or of non-dormant seeds held at unfavorable low temperatures.

Samples which had germinated in the greenhouse after storage in water for various lengths of time at temperatures ranging from approximately −4° C. to 40° C. showed 20° and 30° C. better than lower or higher temperatures for keeping the seeds viable as long as 17 months. It was also demonstrated that the speed of germination of all lots was hastened by a storage period of a month or longer, and the percentage of germination of the less mature lot C was greatly increased by such treatment.

LITERATURE CITED

1. HAIGH, J. C. The propagation of water hyacinth (*Eichhornia crassipes* Solms.) by seed. Trop. Agric. [Ceylon] **94**: 296–297. 1940.
2. HITCHCOCK, A. E., P. W. ZIMMERMAN, HENRY KIRKPATRICK, JR., and T. T. EARLE. Water hyacinth: its growth, reproduction, and practical control by 2,4-D. Contrib. Boyce Thompson Inst. **15**: 363–401. 1949.
3. ———— Growth and reproduction of water hyacinth and alligator weed and their control by means of 2,4-D. Contrib. Boyce Thompson Inst. **16**: 91–130. 1950.
4. PENFOUND, WM. T., and T. T. EARLE. The biology of the water hyacinth. Ecol. Monographs **18**: 447–472. 1948.

ROSE "SPORTS" FROM ADVENTITIOUS BUDS

P. W. Zimmerman and A. E. Hitchcock

Many horticultural varieties of plants are multiplied by vegetative means to avoid genetic variation which often occurs when such species are propagated from seed. Plants derived from normal buds on the stem are like the parent type though occasional abnormal branches arise. These bud "sports" are due to somatic mutations or other variations in the bud. Roses frequently produce bud sports, and these have been of considerable importance in the selection of new varieties. They retain their desirable qualities when subsequently propagated from stem cuttings. New plants derived from root cuttings or adventitious buds on the stem, however, often differ from the parent type. Since adventitious buds on root cuttings are of endogenous origin, it is obvious that the genetic constitution of the internal core (stele) differs from the outer layer which on the stem produces normal buds. It appears, therefore, that new varieties which arise from bud sports are periclinal chimaeras with different genetic constitutions in the different tissues (stele and cortex).

This report involves new varieties of roses arising from adventitious buds on stems and roots and a bud sport of a commercial rose (*Rosa* sp., var. Briarcliff) called to the writers' attention about 1940 by Henry Kirkpatrick, Jr., of this laboratory.

RESULTS AND DISCUSSION

A bud sport appeared on a branch of a Briarcliff hybrid tea rose growing in a greenhouse. One branch differed from the rest of the plant in both leaf and flower characteristics. Briarcliff has a double pink flower and serrate leaves typical of the rose. The sport branch had nearly single pink flowers with crinkled petals and narrow, non-serrate leaves (Fig. 1).

When the sport was propagated from stem cuttings, the new plants were like the original sport branch. When, however, the plants were propagated from adventitious buds arising on root cuttings, the new plants had leaves and flowers resembling Briarcliff variety. Also suckers from underground parts of the stem (presumably adventitious buds on internodes) reverted to Briarcliff type.

In attempting to determine the reasons for reversion to the original parent stock, it was assumed that the sport was a periclinal chimaera resulting from unusual somatic segregation in the bud. The inner core appeared to be Briarcliff while the outer layer was a new mixture of cells. This assumption is strengthened somewhat by the fact that three genera-

Copyright, 1951, by Boyce Thompson Institute for Plant Research, Inc.

tions of root cuttings have continued to produce plants like Briarcliff. All plants from root cuttings (a total of 55) of the sport variety were like the Briarcliff strain. Ten plants, all like Briarcliff, have come from root cuttings of the first reversions. From the latter have come five new plants like Briarcliff, by way of root cuttings. It appears, therefore, that a pure line Briarcliff strain which continues to produce the same variety regardless of the method of propagation can be developed. Stem cuttings have never failed to come true to type.

Three other varieties of hybrid tea rose, Better Times, Souvenir, and one garden type (name unknown), did not come true when propagated from root cuttings.

Better Times (Plant Patent 23), a hybrid tea type selected in 1934 as

FIGURE 1. Leaves of a bud sport of Briarcliff rose (left) and leaves of a plant which was made from a root cutting of the sport.

a bud sport from Briarcliff by J. H. Hill Company, has deep red flowers. It came true to type when propagated from stem cuttings, but when grown from root cuttings the flowers resembled Briarcliff. In one lot of twenty root cuttings one new plant was a climbing rose, resembling neither Briarcliff nor Better Times. This exception militates to some extent against the simple explanation that the adventitious buds from which the new plants developed, arose in the core of a periclinal chimaera and that these resembled only one of the parents.

The climbing rose varied from the parent plant in leaf, thorn, and flower characteristics and habit of growth. It did not bloom continouusly as a hybrid tea rose but resembled the average climbing rose which flowers annually. Clusters of flowers appeared on the terminal part of the main shoots. The buds along the stem became dormant and the plant required two months of cold temperature to resume growth. In fact there were no special similarities to the parent plant.

It is not new to find hybrid tea roses producing climbing types. Hjort (3) reported that Killarney HT, Maman, and Catherine Mermet all gave rise through bud sports to climbing roses. The same author reported that climbing sports were known to revert to the bush form. It will be interesting to test the climbing sport of Better Times to see if its relationship can be detected.

Souvenir (Plant Patent 25 by A. N. Pierson in 1930) arose as a bud sport of Talisman. The latter is said to be a hybrid between Pernet and Ophelia strains. Talisman has given rise, perhaps through somatic segregation, to many bud sports with fairly stable characteristics. When Souvenir, however, was propagated from root cuttings a strain with Talisman-like characters appeared. These have been propagated from both stem and root cuttings without change of characteristics. It appears, therefore, that Souvenir is a periclinal chimaera with a central core of Talisman. Endogenous buds arising from this core in turn produce the pure line Talisman strain. Three generations of root cuttings continued to make the Talisman-like variety.

An unidentified garden type of a deep red hybrid tea rose gave rise to plants with pink flowers when propagated from root cuttings. The buds appear to be of endogenous origin and to arise from the core of a chimaera.

There are many varieties of garden and greenhouse roses with unknown ancestry. Through the use of root cuttings or other means of producing plants from adventitious buds it might be possible to obtain some information on the ancestry of named varieties of roses.

Bateson (1, 2) reported that in *Bouvardia* some double flowering types give rise to single flowers when propagated from root cuttings. A variety called Bridesmaid (double pink) regularly gave rise to a double scarlet when propagated from root cuttings. *Pelargonium* varieties propagated from "suckers" or root cuttings differed from the parent plants in color of the flowers as well as in their phenotypic characteristics. Bateson said that whenever plants grown from root cuttings differ from those grown from stem cuttings it may be inferred that the plant is a periclinal chimaera in which the cortex differs from the central tissue (core).

In the case of rose sports reported herewith only one arose from a natural stem bud of Briarcliff variety. All others came from adventitious buds of stems or roots. Those from the stems arose as suckers from internodal tissue growing underground. With the exception of one case, the adventitious buds on both stems and roots gave rise to the same kind of plants for any given variety. In general, therefore, the adventitious buds appear to be of endogenous origin. No explanation can be given for the exception where a root cutting gave rise to a climbing rose.

SUMMARY

A new kind of rose (sport) appeared on a branch of Briarcliff variety. The new rose flower was single and pink. The parent plant was a double pink. When plants of the sport were propagated from stem cuttings, they continued to show the same characteristics. When plants were propagated from root cuttings involving adventitious buds, they reverted to the original Briarcliff variety.

The variety Better Times came true to type when propagated by stem cuttings but reverted to Briarcliff characteristics when propagated from root cuttings. Also another unidentified red rose produced pink flowers when propagated from root cuttings.

Souvenir variety (yellow) which is known to be a bud sport of Talisman (red) gave rise to Talisman type plants from root cuttings.

It was concluded that the rose varieties described were probably periclinal chimaeras and that plants arising from adventitious buds of endogenous origin were like the variety which constituted the stele (core).

LITERATURE CITED

1. BATESON, W. Root-cuttings, chimaeras and "sports." Jour. Genetics **6:** 75–80. 1916/17.
2. ———— Root-cuttings and chimaeras II. Jour. Genetics **11:** 91–97. 1921.
3. HJORT, P. J. Sporting tendencies of newer vs. older roses. Amer. Rose Ann. **1938:** 50–52. 1938.

A QUANTITATIVE METHOD OF MEASURING RESPONSE OF PLANTS TO GROWTH REGULATORS

A. E. Hitchcock and P. W. Zimmerman

Growth regulators and herbicides have been evaluated in greenhouse and field tests by various methods which have involved application of the test substance in solutions, suspensions, or in solid form to intact plants, decapitated plants, or to cuttings of various species of plants. Results obtained by these methods furnished information on the capacity of a substance to induce formative and herbicidal effects, and the concentrations required to bring about a given response. The quantity of growth regulator applied to an individual test plant was generally not known. When tests were undertaken with the objective of studying the effects of applying two or more growth regulators to the same test plant, it was necessary to develop more precise quantitative methods for treating plants and evaluating the induced responses.

An improved technique for treating intact plants consisted of applying small measured volumes of an aqueous solution of the growth regulator (0.001 to 0.01 ml.) to one or more leaves or leaflets by means of a pipette. The evaluation of results consisted of rating the treated plants according to the magnitude of a given response or, according to all responses, assigning rank values to each plant, and then analyzing the assigned values by statistical rank methods. Dosage response curves based on measured responses on plants treated under a standard set of conditions and procedures were used as a reference for determining quantitatively the influence of any new factor. Results with 2,4-D showed that the concentration of 2,4-D, the number of treated leaflets, and the quantity of 2,4-D (γ per plant) were of increasing importance, in the order named, for increasing the magnitude of the induced responses on tomato.

Since practically all responses induced by growth regulators can be readily evaluated on intact tomato plants, it is believed that the present method with its improved quantitative technique for treating plants, and the use of a rapid statistical procedure for evaluating results, has an advantage over other methods used to date. The present method is about three times as sensitive as the standard lanolin and spray solution methods with respect to the minimum quantity of 2,4-D required to induce a given magnitude of response. The tomato proved more satisfactory as a test plant than snap bean, sunflower, or zinnia, particularly from the standpoint of the number of responses which could be readily seen and easily rated with or without the use of measurements.

Copyright, 1951, by Boyce Thompson Institute for Plant Research, Inc.

This paper describes an improved technique for treating intact tomato plants with 2,4-D and a rapid statistical procedure for evaluating the induced responses. Only limited data relating to combination treatments will be presented, since a more detailed report on the subject is being prepared.

METHODS

Selection of plants. Bonny Best tomatoes (*Lycopersicon esculentum* Mill.) growing in 4-inch pots were used when plants of the same age were 7 to 9 cm. in height. A further selection was made on the basis of the number and size of leaves and particularly with reference to the similarity of leaflets on the fourth leaf. The plants were spaced on the greenhouse bench so that stems and leaves could assume their normal position, and during the 24 hours before and after treatment care was taken to avoid disturbing them while the soil in the pots was being watered.

In addition to tomato, the following species were used for combination treatments: snap bean (*Phaseolus vulgaris* L. var. Tendergreen), zinnia (*Zinnia* sp. pompon type), and sunflower (*Helianthus annuus* L. var. Mammoth Russian).

Treatment of plants. Treatments were carried out between 8:30 and 10:30 A.M. on days when the light intensity was relatively high for the season. Temperatures in the greenhouse ranged from 70° to 85° F. during the day and 68° to 70° F. at night. Aqueous solutions of 2,4-D were prepared from a commercial formulation (Dow Chemical Co.) of an alkanolamine salt mixture (ethanol and isopropanol series) containing 40 per cent 2,4-D acid equivalent (4 lb. 2,4-D per gal.). Dilutions of 0.4 per cent or higher were made on a gravimetric basis with respect to the quantity of 2,4-D acid equivalent required for a given concentration and then made up to volume with distilled water. Lower dilutions were made from a 0.4 per cent solution on a volumetric basis.

The alkanolamine salt of 2,4-D was chosen as a standard solution because of its ready availability on the market and its high solubility in water. Results with other formulations of 2,4-D, including those containing spreaders and other adjuvants, will be discussed in a later report.

Measured volumes of test solution (0.001 to 0.01 ml.) were applied to a tomato leaflet by means of a graduated pipette having a capacity of 0.1 ml. The graduated divisions were equivalent to 0.01 ml. on one type of pipette and to 0.001 ml. on another type. The test solution was deposited on the upper surface of a leaflet by contact with the midrib 5 to 10 mm. above the base. A standard treatment consisted of treating one or more leaflets on the fourth leaf.

Evaluation of responses. The principal responses evaluated on plants treated with 2,4-D were as follows: modification of leaves, stem bending, declination of treated leaf, proliferation of stem tissue (change in color and swellings generally associated with induction of roots), and change in

rate of stem elongation based on height measurements of treated plants as compared with controls. Stem bending and the declination of the treated leaf were measured with a protractor having a radius of one and seven-eighths inches. The increase in height (stem elongation) of test plants was measured in centimeters. Other responses were estimated in terms of several relative magnitudes ranging from a trace to an extreme or maximal degree, each relative magnitude of response being assigned a different numerical value ranging from one to six. The total effect of the treatment was determined by rating the plants according to the combined expression of all responses. There was good agreement between rankings for total response and each individual response.

In view of the tedious procedure used to obtain numerical values for all responses, a more rapid method of evaluation was sought. A method of evaluating the toxic effects of sulfur dioxide based on the rating of treated plants according to their relative degree of injury (8) appeared to be applicable in the present tests for rating the responses induced by 2,4-D on tomatoes. Initial results showed that the individual responses as well as the total effect of the treatment induced by 2,4-D on tomato could be quickly rated merely by arranging the treated plants in one or more rows in the order of an increasing magnitude of any given response. The rating of 30 to 40 treated plants generally required ten minutes or less. Rank values were assigned to each plant, beginning with a rank of 1 for the least response and increasing consecutively to the highest rank value for the most pronounced response. The rank values were used as a basis for statistical analysis (3, 5, 10, 11).

The influence of important limiting factors was determined by arranging the ranks as shown in Tables I to IV inclusive. Relationships between different responses in the same tests, including the agreement of replicates, and in different tests, were determined by means of rank difference correlation coefficients, the calculation of which is a simple, rapid procedure (9, p. 165; 10). Efficient use of rank methods for evaluating responses induced by 2,4-D depends upon a knowledge of the doses required to induce a given magnitude of response on the test plant. The importance of this information for the rating of treated plants may be judged by an inspection of the dosage-response curves in Figure 1. The log scale was used as a matter of convenience for covering a wide range of dosage. It is, of course, necessary to determine dosage-response relationships for each new compound being tested.

When responses involved a wide range of dosage, it became necessary to assign increasingly higher ranks to plants exhibiting responses corresponding to the descending portion of the dosage-response curves. For example, if two treated plants had increased in height by 20 mm., they could be assigned a rank corresponding either to 1 γ 2,4-D on the ascending portion of the curve for increase in height, or to 6 γ 2,4-D which lies on

the descending portion of the same curve (Fig. 1). The presence or absence of proliferation would determine whether either or both plants were assigned the higher or lower ranks. The method of assigning increasing rank values proportional to the decreasing expression of a given response in the high dosage range is shown in Table IV. If this adjustment were not made, some of the treated plants in the high dosage range would have the same ranks as plants in the low range, thus indicating little or no effect of the treatment. This type of adjustment constitutes a special feature of the present method of evaluation since responses corresponding to all portions of a dosage-response curve are utilized in rating treated plants. The absence

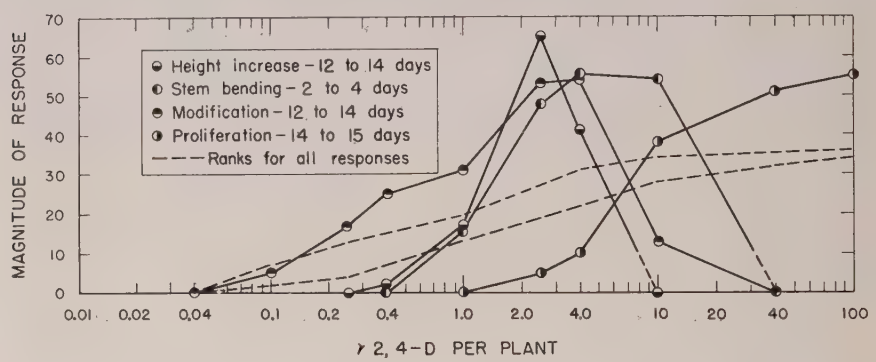


FIGURE 1. Dosage-response curves based on the average responses on tomato in three tests and the corresponding ranks showing the maximum deviations in all tests.

of a response or its lesser expression in the high dosage (descending) range is fully as important for rating plants as any magnitude of response corresponding to the ascending portion of the dosage-response curve.

RESULTS WITH 2,4-D

Rating based on all responses. The influence of concentration and quantity of 2,4-D and the number of treated leaflets on the response of tomato was first determined by rating the plants according to the combined expression of all responses at various time periods after treatment. All of the principal responses, except proliferation, used as a basis for rating the treated plants increased with the dose of 2,4-D up to a maximum and then decreased as indicated in Figure 1 and as illustrated in Figures 2 and 3. Proliferation increased with the dose of 2,4-D up to a maximum. Relative differences in individual responses, such as threshold values, the order in which each response reaches a maximum, and the presence of inhibitive effects associated with a decrease in rate of growth were important criteria for rating treated plants. As will be shown later, the ranks for individual responses were correlated with the ranks for all responses (Table IV).

If doses of 2,4-D high enough to inhibit growth were included, the rating

of treated plants increased with the magnitude of the response up to a maximum response and thereafter the rating continued to increase in proportion to the decrease in response shown on the descending portion of the dosage-response curves (Fig. 1 and Table IV). Plants shown in Figure 2 can serve as examples of how the responses on treated plants were rated on the basis of relative differences and assigned rank values. In Figure 2 A the plants would be ranked 1, 2, 3, 4, 5, 6 from left to right, since this order represents an increase in stem bending, stem elongation, and modification of terminal leaves. Similarly the plants in Figure 2 B would

FIGURE 2. Results obtained with different volumes of 2,4-D solution applied to one leaflet on tomato, showing relation between concentration (%) and quantity of 2,4-D applied to each plant. Increasing amounts of 2,4-D, from left to right, are shown in A and B.

be rated in increasing order from left to right on the basis of increased stem bending for the four plants on the left, and a decrease in modification and in terminal growth for the four plants on the right. The degree of proliferation on stems increases from left to right. A quantitative method of rating treated plants, according to the dose of 2,4-D required to induce a given response, is described in another section.

Evaluation of combined responses. After the treated plants had been rated and assigned ranks, the rank values were used as a basis for determining relationships between concentration and quantity of 2,4-D, the volume of test solution applied per leaflet, and the number and location of the treated leaflets. In one experiment 16 different doses of 2,4-D were ap-

FIGURE 3. Relation between the magnitude of response on tomato and the number of leaflets treated with the same dose of 2,4-D when 0.01 ml. of 2,4-D was applied to one or more leaflets on the fourth leaf. Concentration of 2,4-D was as follows: A. 0.0025; B. 0.01; and C. 0.04 per cent, expressed as γ per plant. Number of treated leaflets in A, B, and C was, from left to right, 0, 1, 2, and 4.

plied in duplicate, making a total of 32 treated tomato plants. When the ranks for combined responses are arranged as shown in Table I, it is seen that they increase with increasing concentration and quantity of 2,4-D and with increasing number of treated leaflets. Duplicate treatments showed good agreement as indicated by the near equality of ranks.

The relative importance of concentration and quantity of 2,4-D and the number of treated leaflets is also shown in Table I, but these relation-

TABLE I

TOTAL EFFECT OF 2,4-D TREATMENT ON BONNY BEST TOMATO AFTER TEN DAYS AS REPRESENTED BY RANKED RESPONSES*

No. of leaflets treated with 0.01 ml. of 2,4-D	Ranks for responses induced by the concentrations of 2,4-D (γ ml.) shown				Rank totals
	7	28	110	440	
1	1, 2 } 1**	4, 3 } 2	10, 9 } 5	15, 15 } 7	59
4†	5, 6 } 3	13, 17.5 } 8	20, 19 } 10	25, 25 } 13	130.5
4††	7, 8 } 4	11.5, 11.5 } 6	23, 25 } 12	27.5, 29 } 14	142.5
16	15, 17.5 } 9	22, 21 } 11	30, 27.5‡ } 15	31‡, 32‡ } 16	196
Rank totals	61.5	103.5	163.5	199.5	—

L.S.D. for rank totals 28 at 5% and 40 at 1% levels

* Rank 1 least and rank 32 greatest response.
** Ranks for the average of duplicates (outside bracket) used in Tables II and III.
† Four leaflets on fourth leaf treated.
†† One leaflet on each of four leaves treated.
‡ Plants killed by treatment.

ships are more readily seen when the same data are rearranged on the basis of average ranks as in Table II which required re-ranking as indicated in Table I, to give 16 ranks. When each of the three factors was held constant while varying the other two, the ranks for response increased considerably more when concentration or number of leaflets was held constant than when the dose of 2,4-D (γ per plant) was held constant. In this case an average increase of 4.8 ranks (col. 6) was obtained for the 14 combinations in which a four-fold increase in the dose of 2,4-D (col. 7) was brought about by a four-fold increase in the number of treated leaflets (col. 8). For example, at a concentration of 27.5 γ per ml. (col. 5), treating 4 leaflets as compared with 1 leaflet (col. 8) resulted in an increase in ranks from 2 to 6 in one case and from 2 to 7.5 in another case (col. 6). These results are to be compared with the smaller average increase of 2.8 ranks (col. 10) for the 11 combinations in which a four-fold increase in dose of 2,4-D (col. 11) was brought about by a four-fold increase in concentration (col. 12).

In the case of treating 4 leaflets on one leaf (col. 9) increasing the concentration from 6.9 to 27.5 γ per ml. (col. 12) caused an increase from 4 to 7.5 ranks (col. 10). If the highest dose (70.4 γ) is omitted, the corresponding average increases were 5.2 ranks for 12 combinations (col. 8) and 3.0 ranks for 10 combinations (col. 12) respectively. Similar results were obtained in the test where 1, 2, or 4 leaflets on the same leaf were treated (Fig. 3).

TABLE II

RELATIVE IMPORTANCE OF CONCENTRATION OF 2,4-D, QUANTITY APPLIED, AND NUMBER OF TREATED LEAFLETS ON TOTAL EFFECT OF THE TREATMENT ON BONNY BEST TOMATO. DATA FROM TABLE I

Responses ranked* according to each of three factors at increasing levels

Quantity 2,4-D				Concentration 2,4-D				No. of treated leaflets			
		Corresponding				Corresponding				Corresponding	
γ per plant	Rank	Concn., γ/ml.	No. of treated leaflets	γ per ml.	Rank	γ per plant	No. of treated leaflets	No.	Rank	γ per plant	Concn., γ/ml.
0.069	1	6.9	1	6.9	1	0.069	1	1	1	0.069	6.9
					3	0.275	4**		2	0.275	27.5
	2	27.5	1		4	0.275	4†		5	1.1	110
0.275	3	6.9	4**		9	1.1	16	1	7.5	4.4	440
	4	6.9	4†								
	5	110	1	27.5	2	0.275	1	4†	4	0.275	6.9
1.1	6	27.5	4**		6	1.1	4**		7.5	1.1	27.5
	7.5	27.5	4†		7.5	1.1	4†		10	4.4	110
	9	6.9	16		11	4.4	16		13	17.6	440
	7.5	440	1	110	5	1.1	1	4**	3	0.275	6.9
4.4	10	110	4†		10	4.4	4†		6	1.1	27.5
	11	27.5	16		12	4.4	4**		12	4.4	110
	12	110	4**		15	17.6	16		14	17.6	440
	13	440	4†	440	7.5	4.4	1	16	9	1.1	6.9
17.6	14	440	4**		13	17.6	4†		11	4.4	27.5
	15	110	16		14	17.6	4**		15	17.6	110
					16	70.4	16		16	70.4	440
70.4	16	400	16								

* Rank 1 represents the least and rank 16 the greatest effect on two plants.
** 0.01 ml. 2,4-D applied to one leaflet on each of four leaves.
† 0.01 ml. 2,4-D applied to each of four leaflets on one leaf.

These results are in line with the trend (Table I) showing that killing occurred with a lower dose (17.6 γ per plant) when a large number of leaflets, namely 16, was treated as compared with the treatment of smaller numbers (4 leaflets). In other tests the minimum lethal dose was 40 to more than 100 γ when applied to one leaflet.

Evaluation of individual responses. The principal responses induced by 2,4-D on tomato within 14 days were as follows, in the order of decreasing sensitivity: modification of leaves, curvature of stem, stem elongation, temporary declination of treated leaf petiole, induction of roots, swelling

and proliferation of stem tissue, permanent declination of leaves, inhibition of terminal growth, and killing. Responses requiring substantially longer than 14 days to evaluate, but which are not included in the present analysis, were the time of flowering and fruit set, development of parthenocarpic fruit, modification in shape of fruit, reduction in rate of development of roots, modification of roots, threshold values for wilting of foliage, and modification of leaves that develop after the resumption of terminal growth following a period of growth inhibition.

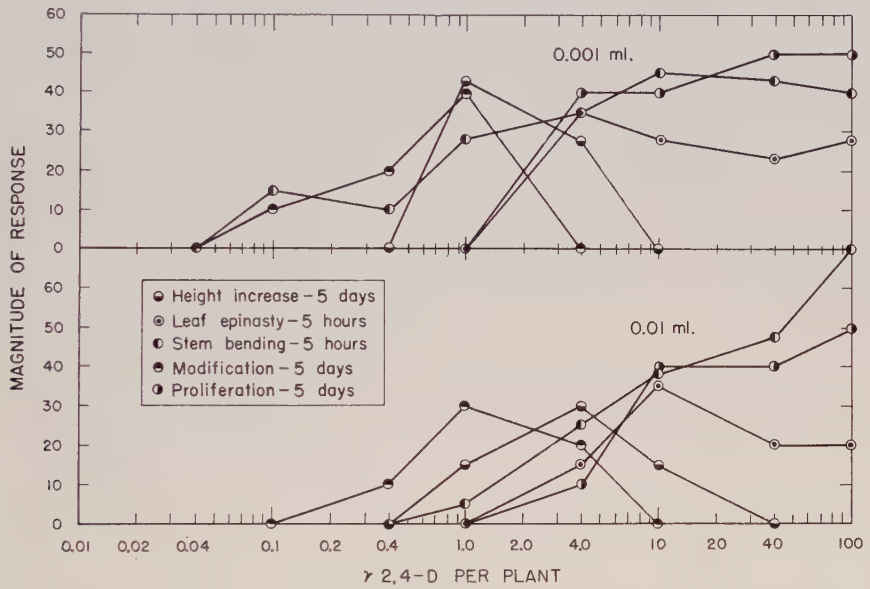


FIGURE 4. Dosage-response curves for responses evaluated five hours or five days after treating one leaflet on tomato with different volumes of 2,4-D solution.

The range of dosage of 2,4-D for inducing four different responses is shown in Figure 1 and Table IV. In view of the differences in threshold values and in the shapes of the dosage-response curves (Fig. 1), a relatively wide range of dosage must be used in order to study the relationship between these particular responses. For example, stem elongation and modification of leaves fell from maximum to zero values in the range of dosage where proliferation continued to increase toward a high level of response. However, all four responses were measurable in the range of 1 to 4 γ per plant within 5 days (Fig. 4), or in 12 to 14 days (Fig. 1).

Individual responses which increased in degree with increasing doses of 2,4-D were compared at the same and at different times after treatment, depending upon the time of appearance and the duration of the response. Declination of the treated leaf was evaluated within five hours after treatment (Fig. 4) and longer lasting curvatures on any leaf were estimated at

the end of 24 hours (Fig. 5 A) or later. Negative changes in rate of stem elongation required more than five days to evaluate. Stem curvature was evaluated at various periods from five hours up to the time the weight of the plant became a limiting factor which was about two weeks. During this period changes in stem curvature were of relatively the same order of magnitude for doses of 2,4-D of 1 to 10 γ per plant, but varied noticeably

TABLE III

CORRELATION OF INDIVIDUAL RESPONSES WITH ALL RESPONSES ON BONNY BEST TOMATO PLANTS TEN DAYS AFTER TREATMENT

No. of leaflets treated	Concn. of 2,4-D, γ/ml.	Ranks for different responses				
		Epinasty leaves, 24 hr.	Stem curvature, 7 days	Proliferation on stem, 7 days	All responses*	
					7 days	10 days**
1	6.9	1	1	1	1	1
	27.5	2	2	4	4	2
	110	9	7	5	6	5
	440	9	6	10	9	8
4†	6.9	4	3	2	3	3
	27.5	5	12	8	8	7
	110	9	9	11	11	10
	440	9	13	13	12	13
4††	6.9	3	4	3	2	4
	27.5	6	9	6	5	6
	110	12	15	12	14	12
	440	15	9	15	13	14
16	6.9	9	5	7	7	9
	27.5	14	14	9	10	11
	110	13	11	14	15	15
	440	16	16	16	16	16
Rank difference correlation coefficient (r')‡		.8794	.8382	.9617	.9647	—

* Including modification of leaves and inhibition of growth.
** Ranks from Table I with split rank values of 7.5 shown here as 7 and 8 respectively.
† Four leaflets on one leaf.
†† One leaflet on each of four leaves.
‡ $r' > .516$ = significant correlation.

with time for doses higher than 10 γ (Fig. 5 A). Eventual recovery to the vertical position occurred below 1 γ. The relations between certain individual responses and between each response and the total effect of the treatment are shown in Tables III and IV and in Figure 1.

The method of ranking individual responses was the same as that used for ranking the total effect of the treatment (Fig. 1 and Table IV), namely, the use of increasing rank values for increasing degrees of response up to a maximal expression, and a continued increase in rank value for a decreasing response associated with inhibition of terminal growth or other high dosage effects. In the case of measured values this meant assigning higher ranks

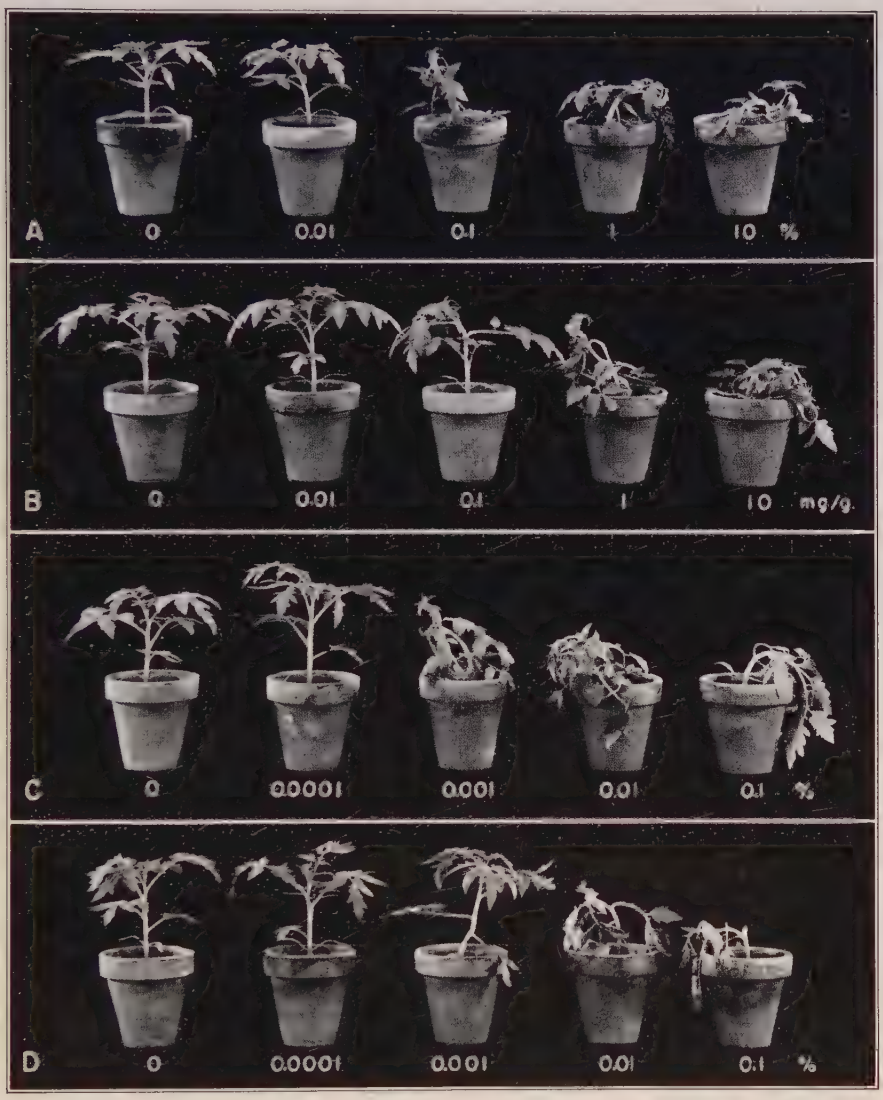


FIGURE 5. Results obtained with four different methods of treating tomato with 2,4-D at a dose ratio of 10, increasing from left to right with controls on left: A. 0.01 ml. applied to one leaflet, starting with 0.01 per cent; B. 10 mg. 2,4-D per g. lanolin applied to base of leaf and adjacent stem, starting at 0.01 mg. per g.; C. 3 ml. spray solution applied to foliage, starting at 0.0001 per cent; D. 20 ml. applied to soil surface, starting at 0.0001 per cent.

to lower numerical values, including zero and negative values, as indicated in Table IV. If this type of adjustment were not made in the method of ranking, responses which were actually closely correlated on the basis of increasing dosage effects and with highly significant rank difference cor-

TABLE IV

RELATION OF INDIVIDUAL RESPONSES TO COMBINED EFFECT OF ALL RESPONSES ON BONNY BEST TOMATO TEN DAYS AFTER TREATMENT WITH 2,4-D

2,4-D, γ per plant	Curvature of stem away from treated leaf		Proliferation on stem		Modification of leaves		Av. increase in height over controls		Combined effect of all responses
	Degree	Rank*	Relative amount	Rank	Relative amount	Rank*	Mm.	Rank*	Rank*
0.01 ml. applied to leaflet									
100	> 5**	14	12	16	0	13.5	−53	16	15.5
40	> 3**	13	9	11.5	0	13.5	−43	15	14
10	63	12	9	11.5	0	13.5	−18	11.5	11.5
4	38	9	4	9	11	10	38	9	9
1	0	4	0	4	7	7	18	6	7
0.4	0	4	0	4	5	4.5	5	2.5	4.5
0.1	0	4	0	4	1	3	− 8	1	3
0.04	0	4	0	4	0	1.5	5	2.5	1.5
0.001 ml. applied to leaflet									
100	>15**	16	10	14	0	13.5	−28	13	15.5
40	> 8**	15	10	14	0	13.5	−38	14	13
10	58	11	10	14	0	13.5	−18	11.5	11.5
4	48	10	5	10	9	8.5	20	7	10
1	25	8	2	8	9	8.5	50	10	8
0.4	0	4	0	4	6	6	25	8	6
0.1	0	4	0	4	5	4.5	10	4.5	4.5
0.04	0	4	0	4	0	1.5	10	4.5	1.5
r' (>.514 is significant for 16 ranks)		.8603		.8412		.9088		.9242	

* Ranks adjusted for high dosage effects as explained in text.

** Change in direction of curvature after extreme initial bending away from treated leaf (see text).

relation coefficients would appear unrelated. For example, the absence of modification due to lack of growth occurred in the range of 10 to 100 γ of 2,4-D (Table IV). If all eight zero values entered in column 7 were assigned equal ranks of 4.5, the correlation would be non-significant ($r' = -.3315$) instead of the highly significant one shown ($r = .9088$). High rank values of 13.5 were assigned to the six plants showing neither modification nor terminal growth, and low rank values to the two plants showing growth but no modification.

The rate of stem elongation in excess of controls reached a maximum in the range 1 to 4 γ per plant. The entire stem from cotyledons to growing

tip was involved (Fig. 6) with a maximal peak from the 12th to 15th internodes and a lesser peak above and below the treated leaf (3rd to 5th internodes). One to three additional leaves developed at the terminals of the treated plants which exhibited maximal elongation. This noticeable and significant increase in height of treated plants involved a significant increase in wet weight, but not in dry weight. The increase in wet weight was due primarily to a higher moisture content of the stems, there being no marked change in the wet weight of leaves, although many of them were abnormally small and noticeably modified. The increased rate of stem elongation was associated with a measurable degree of stem bending below the treated leaf.

Although measurable stem bending preceded measurable declination

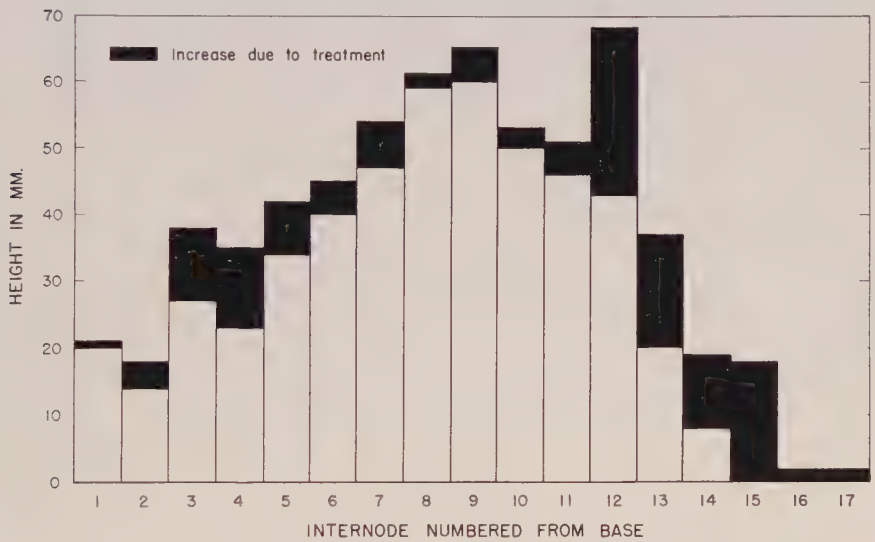


FIGURE 6. Increase in height of tomato showing parts of stem which contributed to increase. The increases shown are the averages for nine plants treated with 1 to 2.5 γ 2,4-D per plant and for nine non-treated controls.

of the treated leaf, these two responses and stem elongation were at near maximum levels of response in the range of 1 to 4 γ of 2,4-D (Fig. 4). This is in contrast to lanolin treatment in which case measurable leaf declination occurred without measurable stem bending when the basal portion of the petiole was treated. It appeared that 2,4-D was translocated more readily from leaflets than from the surface of the petiole. This was further demonstrated by results of tests in which the treatment of the basal portion of the petioles on the second, third, and fourth leaves with 0.1 mg. 2,4-D per gram of lanolin induced less stem bending than a combination of this treatment with application to the adjacent stem. In contrast, stem bending and declination of the treated leaf were much greater when

four leaflets on the fourth leaf were treated with the same lanolin preparation of 2,4-D. However, the treated areas were not comparable in size.

Responses were quantitatively different on plants treated with equivalent dosages of 2,4-D in different volumes of water (Fig. 4 and Table IV). It was originally assumed that 0.01 ml. of a water solution of 2,4-D would spread over an area on the surface of the leaflet equal to about ten times that covered by 0.001 ml. The greater effectiveness of the smaller volume indicated that the foregoing assumption was not correct. Results of one test are shown in Figure 4 for curvature responses occurring in five hours and for other responses occurring in five days. Threshold values for all five responses were lower when 0.001 ml. of test solution was applied to one leaflet on the tomato. Two other tests gave similar results.

Differences in response represented by one of three replicate series of plants in the same test are shown in Figure 2. Where the quantities of 2,4-D were equivalent (0.25, 1, and 4 γ) the concentration for 0.001 ml. was ten times that for 0.01 ml. That the difference in response was probably due to the relative size of the treated area and not to concentration, was indicated by the observed difference in spreading action of 0.01 ml. as compared with 0.001 ml. of the applied test solution. The smaller volume of solution spread quickly into a thin film, covering an equal or greater area on the leaflet than the larger volume which remained in a smaller area, as a spherical-like drop for a period of 30 to 90 minutes, depending upon atmospheric conditions. The increased response obtained with 0.001 ml. of test solution is thus explainable on the basis of a ten-fold increase in quantity of 2,4-D accomplished by a corresponding increase in concentration without decreasing the size of the treated area to the extent of ten times, as would be required for making the treatments comparable. These results show that the size of the treated area is an important limiting factor when treating one leaflet, the same as it was shown to be when treating more than one leaflet.

Dosage-response curves based on measured responses in three different tests appear in Figure 7. The curves are similar in shape for a given response. They show relatively little variation according to dosage in the case of modification and proliferation, but for stem bending the variation is much greater. However, the agreement between tests is considered reasonably good for results based on the evaluation of only two or three plants per treatment. The corresponding maximum deviations of ranks for all responses in these three tests also show good agreement (Fig. 1).

As an example of how plants treated with unknown doses of 2,4-D may be evaluated quantitatively, the responses on certain plants in Figure 3 C can be compared with the dosage-response curves in Figure 1. The approximate dose of 2,4-D is estimated as follows. The plant on the right (Fig. 3 C) shows pronounced stem bending and proliferation which places the dose not in excess of 20 γ (Fig. 1), and the absence of modification and

terminal growth places it above 10 γ (Fig. 1). The plant in question received a dose of 16 γ 2,4-D (Fig. 3 C). In the case of the second plant from the right, a small amount of modification and terminal growth and considerable proliferation places the dose close to, but below, 10 γ (Fig. 1). This plant received a dose of 8 γ 2,4-D (Fig. 3 C). The third plant from the right shows noticeable stem bending and a marked curled leaf type of

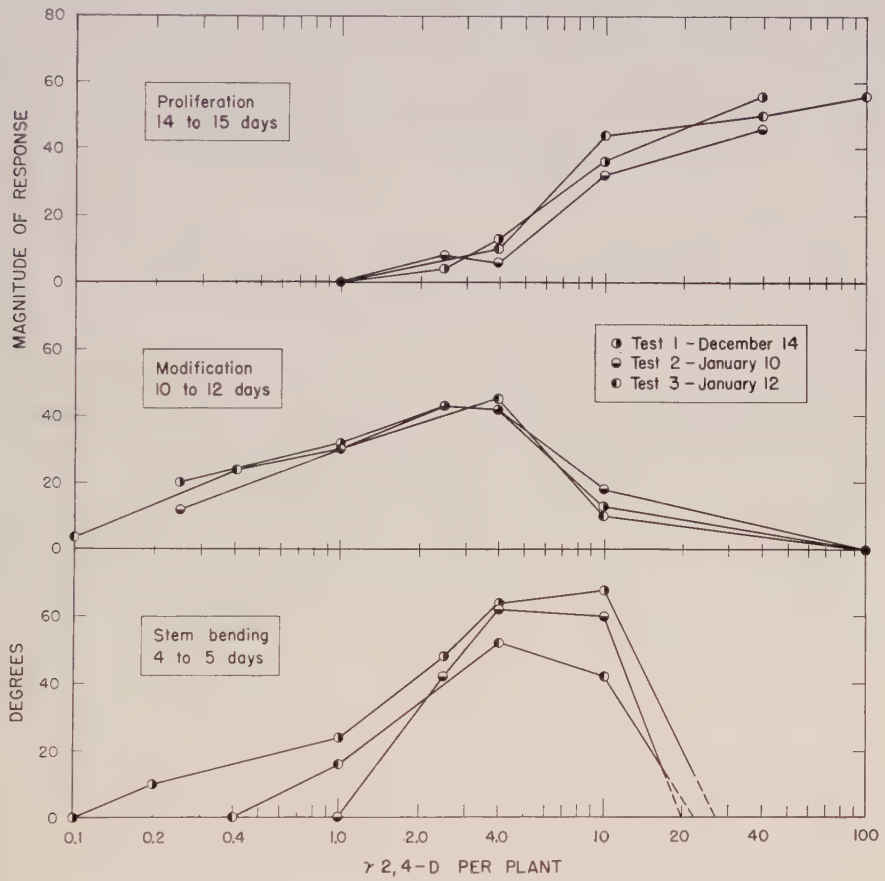


FIGURE 7. Dosage-response curves for three responses on tomato in three different tests.

modification associated with an optimal increase in stem elongation that places the dose between 2.5 and 4 γ (Fig. 1). A dose of 4 γ 2,4-D was applied to this plant (Fig. 3 C). The nine treated plants in Figure 3 were originally ranked according to an increasing degree of response from left to right in each row and from top to bottom in the following order: 1, 2, 4; 3, 5, 7; and 6, 8, 9.

A comparison of the present technique for treating tomato plants with the standard lanolin, spray solution, and soil application methods was

made on the basis of ranked responses (Fig. 8). The appearance of the treated plants after 24 hours is shown in Figure 5. All treated plants were ranked according to the increasing magnitude of the responses ten days

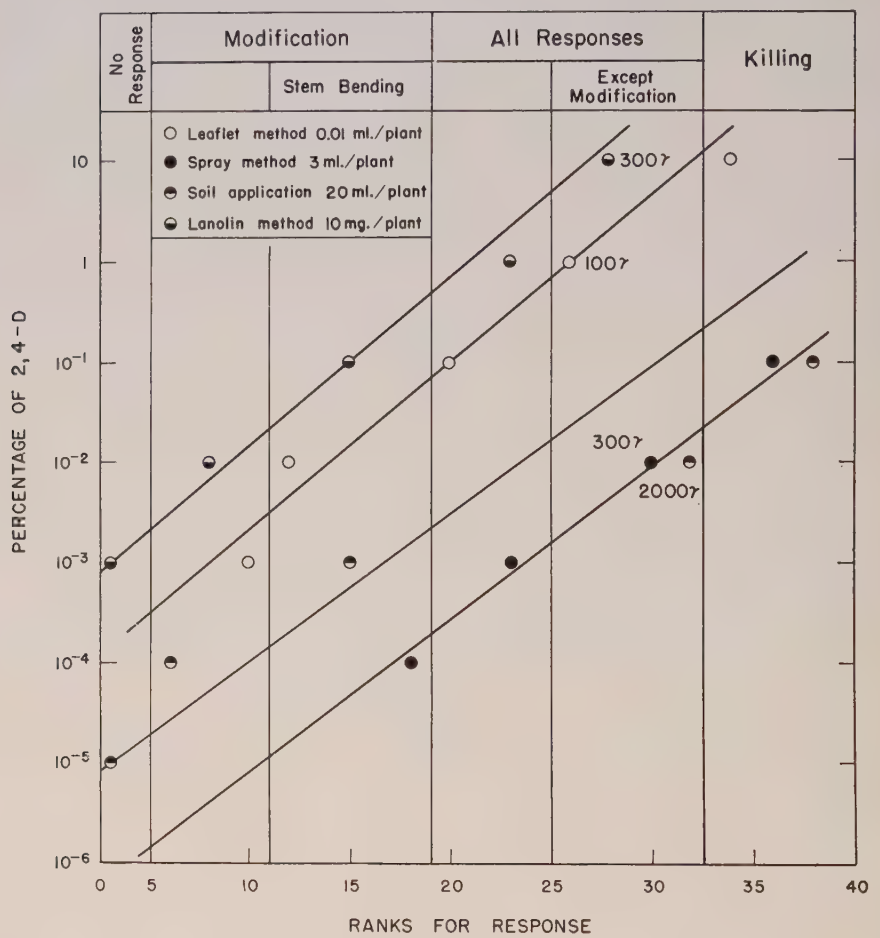


FIGURE 8. Relative efficiency of four different methods of treating tomato with 2,4-D based on responses ranked 10 days after treatment. Doses of 2,4-D required by different methods to induce a given response are shown in the fifth column. The relative differences were the same for other magnitudes of the responses.

after treatment. When the ranks were arranged according to concentration and magnitude of response as shown in Figure 8, the slopes of the dosage-response curves were approximately the same with the possible exception of the soil application in which case the results were much more variable. Since the dose of 2,4-D was known for each method, the ratio between the applied dose by other methods, as compared with the leaflet method, was

considered a measure of the relative efficiency of the other methods. On this basis the technique of applying 0.01 ml. of 2,4-D to one leaflet on a tomato was about three times as efficient as the lanolin and spray solution methods, and about 20 times more efficient than the soil application method.

APPLICATION OF TWO GROWTH REGULATORS TO THE SAME PLANT

Several combinations of two growth regulators have been applied separately and as mixtures by various techniques to tomato and other test plants, and also to cuttings of different species of plants. Indoleacetic acid (IA), 1-naphthaleneacetic acid (NA), and 2,4-D were applied as lanolin preparations in the three possible combinations of 2-substance mixtures of equal parts of each to tomato plants. On the basis of declination of the treated leaf in three hours, IA and NA and IA and 2,4-D were approximately equivalent and additive at near threshold concentrations, but the mixtures of NA and 2,4-D gave generally lower declination values and greater variability in replicate treatments. IA and NA had equivalent threshold values which were one-tenth that for 2,4-D.

When 2,4-D was applied to tomato at the rate of 10 γ per plant, the treatment caused marked proliferation but little or no modification since terminal growth was inhibited (Fig. 1). A dose of more than 100 γ IA was required to induce noticeable proliferation, yet the application of 10 to 100 γ IA in combination with 10 γ 2,4-D caused a marked reduction in proliferation as compared with that induced by 10 γ 2,4-D used alone. Furthermore, the combination treatment resulted in the growth of noticeably modified leaves. These results indicated that IA had reduced the effectiveness of 2,4-D by 2 to 4 times. The reduction was greater when mixtures of IA and 2,4-D were used as compared with applying IA and 2,4-D on separate leaflets.

Tests with lanolin preparations and aqueous solutions of 2,4-D and of 2,3,5-triiodobenzoic acid (TIB) showed either additive or synergistic effects for stem bending and for inducing the growth of axillary shoots on tomato. Additive effects illustrated for stem bending in Figure 9 show that increasing the concentration of either 2,4-D or TIB caused increased bending regardless of whether the two solutions were applied separately to different leaflets or as mixtures. When used alone, 2,4-D caused little or no stem bending at a concentration of 10 to 20 mg. per liter. Likewise, TIB was inactive for stem bending at concentrations of 200 to 400 mg. per liter. At higher concentrations each substance was active for inducing stem bending, but even in the higher range, combination treatments were additive. The additive effects were also evident when the treated leaflets were cut off at various time intervals, ranging from 15 minutes to 24 hours after treatment. The latter results are similar to those obtained with an application of 0.01 ml. of a 0.01 per cent solution of 2,4-D to a tomato leaf-

let in showing that in from 15 minutes to several hours, sufficient 2,4-D entered the rest of the plant to induce rooting and modification responses on cuttings made from stems and leaves of the treated plant.

FIGURE 9. Additive effect on stem bending resulting from application of 2,4-D and TIB solutions to leaflets on fourth leaf (left side) of tomato. A. and C. Doses of 2,4-D shown (mg. per liter) were applied to separate (opposite) leaflets. B. Mixture of equal parts of both substances applied to each of two opposite leaflets.

Combination treatments with 2,4-D and the diethanolamine salt of maleic hydrazide (obtained from Naugatuck Chemical Division) induced additive and antagonistic effects. Doses of 100 to 400 γ of maleic hydrazide (M.H.) and from 10 to 40 γ of 2,4-D per plant disrupted apical dominance in tomato. In one case (10 γ of 2,4-D and 400 γ of M.H.) the continued

growth of four axillary shoots was associated with inhibition of terminal growth for a period of more than seven weeks. An additional shoot at the fifth node, and nearest to the stem terminal, grew to a length of three inches and then stopped, after forming an abnormal type of terminal which concealed the growing tip. Secondary axillary shoots started growing from this particular axillary shoot. The four axillary shoots below continued growing with no evidence of an apical dominance factor being transported from one shoot to another through the main stem of the plant. However, each of these shoots exhibited its own apical dominance system since the axillary buds had not developed.

Evidence that both 2,4-D and M.H. had been present in the four shoots at some time during their growth from the bud stage, was furnished by the presence of modified leaves. The lower one to three leaves exhibited patterns typical for M.H.; the mixed patterns on the middle leaves were caused by the presence of both substances; and the upper leaves exhibited patterns of the 2,4-D-induced type including the dark bluish-green color of the foliage. Plants treated only with M.H. developed fewer and slower growing axillary shoots of light green color similar to non-treated control foliage. The lower one to three leaves were abnormally small and noticeably modified, but those above were of about normal size and the leaflets were of nearly normal shape. During the same period of about six weeks plants treated only with 2,4-D did not form axillary shoots below the one normally located above or below the flower cluster. Later on, modified axillary shoots appeared, indicating that 2,4-D had been present in the axillary buds.

One of the tomato plants which received the same dose of M.H. (400 γ) but four times the dose of 2,4-D used previously (40 γ) developed axillary shoots at the cotyledonary and first nodes at the same time terminal growth was resumed. This occurred after four weeks of inhibition of growth on the entire plant. Terminal and axillary shoot growth was active, and the modified foliage was mainly of the 2,4-D type except for the one to three lower leaves which were of the M.H. type. Similar results were obtained with lower doses of each substance (10 γ 2,4-D and 100 γ M.H.) except that the rate of growth of terminal and axillary shoots was much slower. In this case the apical dominance inhibitor was not transported from the main shoot to the axillary buds which grew from the adjacent lower nodes without inhibiting each other.

In other tests with combination treatments of 2,4-D and maleic hydrazide it was observed that the latter substance decreased or prevented the development of rooting, swelling, and proliferation which was induced by the same doses of 2,4-D when used alone. Similar results were obtained on sunflowers and zinnia. The 2,4-D type of modification was reduced by maleic hydrazide on snap bean. Combination treatments with NA and

2,4-D on tomato resulted in the production of a greater number of root projections associated with extensive areas of only moderate proliferation than was obtained with either substance used alone. Furthermore, the root initials continued to grow out from the stem to a longer length, regardless of the degree of fasciation, as compared with those induced by 2,4-D. Combination treatments of NA and 2,4-D appeared to be additive for stem bending but antagonistic for stem elongation. When used alone, NA did not cause an increase in stem elongation as was true for 2,4-D. The action of 2,4,5-T on rooting was similar to that of NA when used alone and in combination with 2,4-D. This type of rooting response, like the induced growth and development of axillary shoots, is a qualitative difference. The fact that qualitative as well as quantitative differences can be detected and evaluated makes the present method much more flexible than other test methods which are designed to determine only quantitative differences.

Most of the combination treatments which induced unusual effects resulted in greater variation among replicates than was obtained with either substance used alone. Thus most of the unusual effects appear to be critical with respect to the doses of the substances which induce them.

DISCUSSION

Results with the improved method of measuring the response of tomato to 2,4-D showed that under a wide range of conditions there was a consistently close relationship between the dose of 2,4-D (γ per plant) and the magnitude of the induced responses. The responses evaluated by means of rank methods showed close agreement regardless of whether based on measurements or on relative differences arrived at by rating treated plants. This made possible the use of rank methods in conjunction with dosage-response curves as a means of determining quantitatively the influence of new factors on the magnitude of the responses. A change in the magnitude of responses was nearly the same when the dose of 2,4-D per plant was varied by either changing the concentration of 2,4-D or the number of treated leaflets. However, when the volume of applied solution was reduced from 0.01 to 0.001 ml. with a corresponding increase in concentration of ten times, the magnitude of responses was consistently greater instead of being equivalent on the basis of an assumed reduction of ten times in the size of the treated area. Thus there appears to be a limit to the reduction in size of the volume of applied solution which will result in a proportional decrease in the size of the treated area.

Regardless of whether treated plants were rated for individual responses or for the total effect of treatment based on all responses, more than one response was used as a basis for the evaluation. For example, in rating plants for modification, the relative amount of proliferation was used as a guide for assigning ranks to plants which had about the same magni-

tude of modification. In this case the plants showing the greatest amount of proliferation were assigned the highest ranks for modification. Similarly, the relative amount of proliferation was used as a guide for differentiating between plants showing about the same change in height. Thus for plants of about the same height, those showing the greatest amount of proliferation were assigned the highest ranks for increase in height.

It is to be observed that response values on the ascending portion of the curves for modification and increase in height can be the same as those on the descending portion (Fig. 1). However, other responses (proliferation and curvature of stem and leaves) induced by the higher doses of 2,4-D (5 to 10 γ) were sufficiently different to be readily distinguishable from the responses induced by lower doses of 2,4-D (0.5 to 1.5 γ). These and other differences were also used as a basis for determining unknown doses of 2,4-D applied by different methods to tomato plants. By selecting the response values on the dosage-response curves (Fig. 1) which corresponded to those on the treated plant, the equivalent dose of 2,4-D could be read directly from the abscissa values (γ 2,4-D per plant). Data in Figure 8 were derived in this way. The results (Fig. 8) show that for inducing a given magnitude of response on tomato, the leaflet method required application of the smallest quantity of 2,4-D.

Although the lanolin and spray methods of treating tomato were shown to be less sensitive than the present method, the relationship between induced responses and the dose of 2,4-D was essentially the same in all three cases. In view of the much greater variability in results with soil applications, it is not certain whether the dosage-response curves for this method are similar to those for the other methods.

The similarity in results obtained with lanolin preparations and aqueous solutions of 2,4-D indicates that lanolin does not improve penetration or serve as a reservoir of 2,4-D. However, the results serve to emphasize the importance of using the dose of 2,4-D per plant as the basis for comparing the results obtained by different methods. This is in line with the generally accepted practice of recommending the use of herbicides on the basis of the quantity per acre rather than on the basis of the concentration of the applied solution.

The importance of the volume of applied test solution and the location and relative size of the treated areas indicate that some improvement in the lanolin method might result from treatment of a smaller area, particularly if the leaflets were treated. From the standpoint of a routine procedure for comparing the activities of different growth regulators the present method is more rapid with respect to the preparation of a dilution series.

The greater effect resulting from an increase in the number of treated leaflets has a direct bearing on phytotoxicity. Since the lethal dose of 2,4-D decreased with increasing number of treated leaflets (Table I), it seems likely that increasing the number of treated areas on a leaflet would

also decrease the lethal dose. In either case a point would no doubt be reached when any further increase in number of treated areas would cease to show a proportional decrease in the lethal dose of 2,4-D. Thus complete coverage of a tomato plant with a solution of 2,4-D applied as a spray may not always be essential, particularly with relatively high concentrations, as was shown to be the case for water hyacinth (7).

Modification of leaves appears to be the most sensitive detectable response to 2,4-D that can be measured quantitatively. Since the leaf area of a modified leaf is reduced rather than increased, the effect appears to be inhibitory. This is in opposition to the generalization that low doses of growth regulators stimulate and high doses retard or inhibit. It is of interest that a low dose of 2,4-D which causes mild modification of leaves can cause this inhibitory effect without exerting a stimulatory effect, such as cell elongation, in the treated leaf or in the stem. This is in contrast to higher doses which induce optimal cell elongation responses in the treated leaf and in the stem but cause the maximal reduction of leaf area on modified leaves.

Results obtained with combination treatments show that both qualitative and quantitative differences are involved. The consistently greater variability of results obtained with the use of 2,4-D in combination with certain other growth regulators shows that the ratio between the doses is critical. It is also an indication that the two substances are probably affecting the same chemical systems in the tissue in some cases but not in others. In the case of disrupted apical dominance it would appear that maleic hydrazide and 2,4-D affect different chemical systems in the plant. The present method of treating plants appears to be particularly suitable for studying the effects of combination treatments with growth regulators or for determining the effect of various adjuvants on a given growth regulator (6).

The additive and antagonistic effects induced on tomato by combination treatments should not be confused with anti-auxin or auxin antagonistic effects ascribed to TIB by other workers (1, 2, 4) which it is believed do not apply to the present results. TIB was considered an auxin antagonist when applied in combination with IA in the *Avena* test, since curvatures were reduced as compared with the use of IA alone (4). When applied to soybean, TIB did not induce flowering in long-day plants although a greater number of flower buds appeared on the treated short-day plants. It was assumed without measurements that the auxin level was reduced in plants treated with TIB. Non-treated vegetative and flowering soybean showed no consistent or significant difference in auxin level. Likewise, no difference in auxin level was found in long- and short-day *Xanthium* plants (2, p. 623).

When NA was applied to photoinduced short-day *Xanthium* plants, flowering was suppressed without causing a change in auxin level in the

treated plants (1, 2). The assumed anti-auxin effect of NA in suppressing flowering in short-day *Xanthium*, its counteraction by use of TIB in combination with NA, and the induction of flowering in long-day *Xanthium* (but not in long-day soybean) treated with TIB were not supported by data showing that the auxin level had changed either in treated or control short- and long-day *Xanthium* plants or cuttings. These results indicate that there was no relation between the cell-elongating activity of NA and TIB in the *Avena* test and the pronounced regulating effects induced by these two substances in *Xanthium* and soybean, or induced by TIB in tomato (12). Nevertheless, it was concluded, contrary to the findings, that flowering in *Xanthium* may be caused by a decrease in the auxin level in the plant brought about by short photoperiods or by applied anti-auxins (1, p. 627). This assumption was considered to be in agreement with the conclusion of Galston (4, p. 359), that TIB "possesses anti-auxin activity of some sort," even though it was not demonstrated by the results with soybean.

Regardless of whether TIB causes a change in level of extractable substances that are active in the *Avena* test, this could not in itself constitute proof that the substances showing activity on *Avena* played any important part in flowering of the plant in question. In view of the remarkable growth-regulating properties of TIB, other substituted benzoic acids, and many substituted phenoxy acids, the question of whether these growth regulators exhibit cell-elongating activity in the *Avena* test is of minor importance (12, pp. 495, 496), and the same holds true for other test objects used specifically for measuring cell-elongating activity.

SUMMARY

A technique for treating tomato plants with growth regulators consisted of placing important factors on a quantitative basis with respect to the concentration and quantity of the applied growth regulator, the volume of test solution used, and the location and relative size of the treated area. For a given volume of applied test solution (0.001 to 0.01 ml.), the concentration of 2,4-D, the number of treated leaflets, and the dose of 2,4-D (γ per plant) were of increasing importance, in the order named, for increasing the magnitude of the induced responses on tomato.

Evaluation of induced responses consisted of ranking measured responses or of arranging the treated plants in the order of increasing magnitude of any given response, or according to all responses, assigning rank values to each plant, and then analyzing the assigned values statistically by rank methods. Ranked responses in different tests showed good agreement when compared quantitatively by use of reference points on standard dosage-response curves.

The method of rating plants in a continuously increasing order made use of the presence or absence of responses corresponding to all parts of

dosage-response curves, including decreasing, zero, and negative values in the high dosage range.

The decreasing order of sensitivity of responses in the range 0.04 to 100 γ per plant was: modification of leaves, curvature of stem, increase in height (stem elongation), temporary declination of treated leaf petiole, induction of roots, swelling and proliferation of stem tissue, permanent declination of leaves, inhibition of terminal growth, and killing.

Other growth regulators (including TIB) used in combination with 2,4-D induced additive and antagonistic effects on tomato, sunflower, snap beans, and zinnia. Combination treatments applied to tomato also induced responses which were qualitatively different, particularly with respect to rooting (NA and 2,4-D, and 2,4,5-T and 2,4-D) and disruption of apical dominance both with and without active terminal growth (maleic hydrazide vs. 2,4-D). The reason why these effects and some of the effects on flowering reported by other workers cannot be considered anti-auxin or auxin antagonistic effects, is discussed.

On the basis of the minimum quantity of 2,4-D required to induce a given response, the efficiency of the present method was about three times greater than the standard lanolin or spray solution methods, and about 20 times more efficient than the soil application method.

LITERATURE CITED

1. BONNER, JAMES. Further experiments on flowering in *Xanthium*. Bot. Gaz. **110**: 625–627. 1949.
2. BONNER, JAMES, and JOHN THURLOW. Inhibition of photoperiodic induction in *Xanthium* by applied auxin. Bot. Gaz. **110**: 613–624. 1949.
3. FRIEDMAN, MILTON. The use of ranks to avoid the assumption of normality implicit in the analysis of variance. Jour. Amer. Statis. Assoc. **32**: 675–701. 1937.
4. GALSTON, ARTHUR W. The effect of 2,3,5-triiodobenzoic acid on the growth and flowering of soybeans. Amer. Jour. Bot. **34**: 356–360. 1947.
5. HITCHCOCK, A. E., and P. W. ZIMMERMAN. Methods of rating the root-inducing activity of phenoxy acids and other growth substances. Contrib. Boyce Thompson Inst. **14**: 21–38. 1945.
6. ———— Activation of 2,4-D by various adjuvants. Contrib. Boyce Thompson Inst. **15**: 173–193. 1948.
7. HITCHCOCK, A. E., P. W. ZIMMERMAN, HENRY KIRKPATRICK, JR., and T. T. EARLE. Growth and reproduction of water hyacinth and alligator weed and their control by means of 2,4-D. Contrib. Boyce Thompson Inst. **16**: 91–130. 1950.
8. SETTERSTROM, CARL, and P. W. ZIMMERMAN. Factors influencing susceptibility of plants to sulphur dioxide injury. I. Contrib. Boyce Thompson Inst. **10**: 155–181. 1939.
9. SNEDECOR, GEORGE W. Statistical methods applied to experiments in agriculture and biology. 4th ed. 485 pp. The Iowa State College Press, Ames, Iowa. 1946.
10. WILCOXON, FRANK. Some rapid approximate statistical procedures. 12 pp. Amer. Cyan. Co., Agric. Chem. Div. New York, N. Y. [1947].
11. ———— Some rapid approximate statistical procedures. 16 pp. rev. ed. Amer. Cyan. Co., Agric. Chem. Div. New York, N. Y. 1949.
12. ZIMMERMAN, P. W., and A. E. HITCHCOCK. Flowering habit and correlation of organs modified by triiodobenzoic acid. Contrib. Boyce Thompson Inst. **12**: 491–496. 1942.

FLOWERING HABITS AND FERTILITY OF SOME CINCHONA SPECIES IN GUATEMALA

CLYDE CHANDLER

Interest in the culture of *Cinchona* in the western hemisphere was reawakened during World War II when the United States was deprived of shipments from Java at a time of unprecedented demand for quinine. Several corporations and individuals made plantings in Central America. The United States government cooperated by sending scientists to Guatemala to study the problems of propagation, culture, and disease (1). Merck & Co., Inc., which had expanded its plantings of *Cinchona*, inaugurated a research program to increase production by selection of clones and by intraspecific and interspecific hybridization of *C. Ledgeriana* Moens., *C. Calisaya* Wedd., and *C. succirubra* Pav. The writer conducted investigations on this project primarily at the Finca El Naranjo at Chicacacao, Such., Guatemala, from August, 1944 to March, 1947.

In the course of the breeding work it was necessary to study the flowering habits of the various types of trees in all three species. The flowering habits, and the results of self- and cross-pollinations of the various clones available for breeding purposes, are included in this report. Since data on the progeny derived from these studies will not be available for some time, this report must be considered as preliminary in nature until final records may be taken on their quinine content, vigor, and resistance to disease.

Approximately 3,000 clones had been assembled and classified by the time the writer began these studies in 1944. Three distinct species were available: *C. Ledgeriana*, *C. Calisaya*, and *C. succirubra*. In addition, there were clones that appeared to be interspecific hybrids that could not be placed definitely in any of these species. These are referred to in this paper as ledger hybrids or succirubra hybrids, depending upon which species they most closely resembled. Those with truly intermediate characters are simply called hybrids. These various clones were classified as to content of quinine, and other alkaloids, bark thickness, "graftability," style length, disease resistance, vigor, and field location.

For the purposes of the breeding program, it seemed desirable to disregard the very low yielding clones and select only the high yielding ones for further study. Of the total 1,837 Guatemalan clones of *Cinchona*, whose quinine content justified further study, 82 were selected for the present work. Three additional clones of low quinine content but of unusual vigor were used in several combinations.

Copyright, 1951, by Boyce Thompson Institute for Plant Research, Inc.

MATERIALS AND METHODS

Flower behavior. For the studies on flower behavior large flower buds were tagged and observed at three-hour intervals until anthesis was completed and styles had fallen. On April 26 when trees of *C. Ledgeriana* were in heavy bloom two clones of this species were selected for observations. One clone, H1256, has short-styled flowers while the other clone, H1222, has long-styled flowers. At 3 P.M. 50 tags were placed on buds about to open on three different trees of each clone. On June 19 at 6 A.M. 50 tags were placed on buds about to open on two of the trees for each clone in order to determine whether there might be both day and night opening of cinchona flowers.

Similar observations were made for the flower behavior of *C. Calisaya.* Fifty flowers were tagged on each of two trees of the short-styled clone, C1219, and on each of two trees of the long-styled clone N1634. Flowers were tagged at 6 P.M. July 11. The time of tagging the flowers was delayed three hours because of the slightly extended time of the daily rain. It may be mentioned here that the flowering season of cinchona coincides with the rainy season in Guatemala. Observations were made on the same number of flowers tagged the following morning at 6 A.M.

Fifty buds on one tree of short-styled *C. succirubra*, P381, and 50 buds on one tree of long-styled type, P380, were tagged at 6 A.M. on July 25. An equal number of buds were tagged on the same trees at 6 P.M. of the same day. All flower observations on this species were made on trees growing on Finca Panama, near Guatalon.

Breeding. All pollinations were fully controlled. At the time the first bud on an inflorescence was about to open a wire frame was placed over the entire inflorescence and wired to the lower part of the branch. The frame was then covered with a nylon bag open at both ends. Cotton was placed around the branch at the base of the wire frame and the bag was tied in position over the cotton. The other end of the bag was drawn up, folded over, and tied in position. Nylon was used because it dried very quickly after the heavy daily rains. When the first flower opened, the bag was opened from the upper end and rolled back over the wire frame and the flower was pollinated. All clones used in breeding were tested for self-fertility before hybridization pollinations were made.

In 1945 all pollinations were recorded at the time they were made. All capsules resulting from these pollinations were collected and the number of seeds per capsule ascertained. Complete records also were obtained on all failures. If, after pollination, there was no enlargement of the ovary and it withered and fell, the result was recorded as a failure.

The breeding in 1946 was done on a commercial rather than on a scientific scale. However, all pollinations were fully controlled as in 1945. All flowers in a bag were crossed with one clone as a pollen parent. Capsules were collected and as soon as all capsules from a combination were mature

the seeds were collected and weighed. No records were kept for number of seeds per capsule or number of pollinations made.

Due to transportation difficulties and the limited amount of time for the succirubra breeding, one bag was devoted to the pollinations of a single combination. All flowers were tagged and no untagged flowers developed capsules in the bags. Capsules dehisced and seeds fell to the bottom of the bags. The exact number of capsules obtained and number of seeds in each was not determined. The seeds were removed from the bags and weighed.

In 1945 a small greenhouse was built. The seeds obtained as a result of the 1945 breeding program were planted in flats containing sphagnum moss and were watered with a nutrient solution. This method for germinating *Cinchona* seeds was used and found satisfactory at the U. S. Plant Introduction Garden at Glendale, Maryland, and is well described by Morrison (6).

Colchicine. Both seeds and seedlings of cinchona were treated with colchicine in an effort to produce polyploid plants.

EXPERIMENTAL RESULTS

Flower Behavior

The inflorescence of *Cinchona* is a panicle and the terminal flower of every lateral usually opens first as reported by Feenstra-Sluiter (5). However, this is not always the case. The flowering of one panicle has been observed to extend over a considerable period of time. For example, in one inflorescence observed during the course of the present investigations, the first flower opened on July 10 and the last one on August 5. In this particular case, the peak of flowering was reached between July 18 and July 27. The largest number of flowers in this inflorescence opening on any one day was 37 on July 26. There were 326 flowers in this one panicle.

Preliminary to any extended hybridization trials, it becomes necessary to examine the flower structure and behavior. Darwin (2), in 1877, studied dried specimens of *C. Micrantha* Ruiz. & Pav. from Kew and described long- and short-styled flowers of this species. For the *Cinchona* growing in Guatemala, style length has been recorded by other workers for 1,040 clones. Of these, 504 (approximately 48 per cent) have flowers with long styles and the remainder have short-styled flowers. No plant was observed with flowers of both types or with an intermediate style form.

C. Ledgeriana. It will be noted (Fig. 1) that opening of short-styled flowers tagged at 3 P.M. on three different trees began about 9 P.M. the same day and reached a maximum at midnight, nine hours after the large bud stage. Opening continued, however, until 3 P.M. of the following day. Flowers of the long-styled clone opened somewhat earlier than those of the short-styled type. Most of the former tagged at 3 P.M. were open six hours later.

Other large flower buds of both short- and long-styled types were tag-

ged at 3 A.M. to determine whether there might be a set of flowers opening during the day as claimed by Feenstra-Sluiter (5). Most of these did not open until 9 P.M., requiring 15 hours to open as compared with six to nine hours as described above. Thus it has been demonstrated that most of the flowers of *C. Ledgeriana* open at night in Guatemala and not at sunrise as reported by Engelbeen (4).

C. Calisaya. Most of the flowers of the long-styled clone of this species open about midnight, but those of the short-styled clone seem to belong

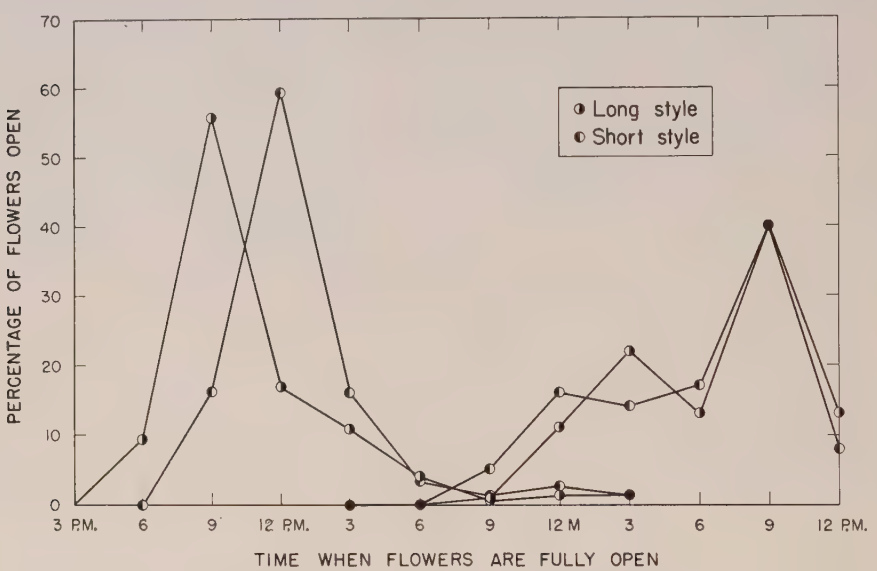


FIGURE 1. Time of flower opening in long-styled and short-styled types of *Cinchona Ledgeriana*. Each graph based on observations of 150 flowers. Flowers in the large bud stage were tagged at 3 P.M. or at 3 A.M. for observation.

to two types—day opening and night opening. The progress of flower opening is shown in Figure 2.

C. succirubra. On the short-styled plant only ten of the 50 buds tagged at 6 A.M. and five of those tagged at 6 P.M. opened. The others never opened and the closed corollas fell leaving the style exposed for pollination. A few more of the buds on long-styled trees opened. Nineteen of the buds tagged at 6 P.M. opened from midnight to 6 P.M. of the following day, while 38 buds tagged at 6 A.M. opened during the evening from 6 to 9 P.M. Hybridization pollinations showed that the stigmas of the succirubra flowers were receptive though the flowers never opened. Further observations should be made for other clones of succirubra in order to determine whether their flowers usually behave in this manner.

The length of time the flowers remain open and in good condition is im-

portant in any breeding program. A summary of the average number of hours required for the various stages of anthesis for flowers of the three cinchona species is given in Table I.

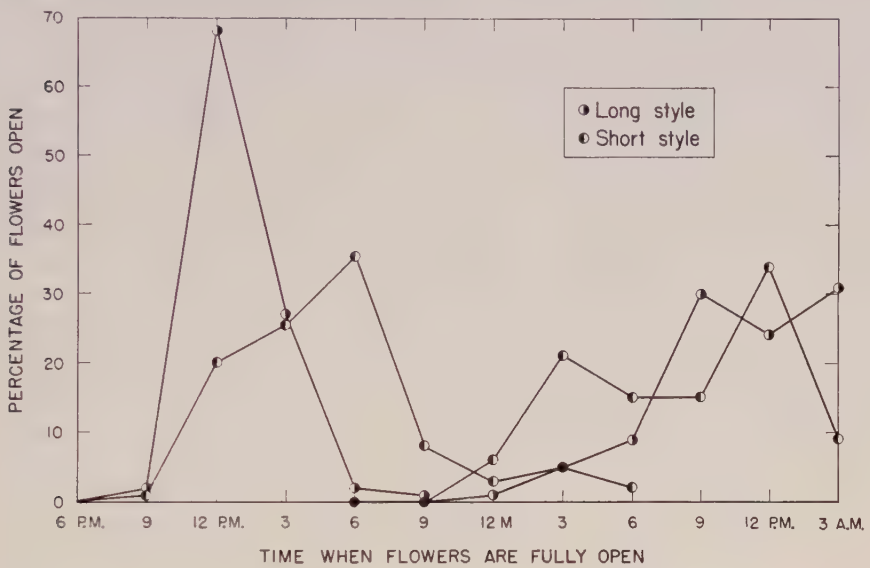


FIGURE 2. Time of flower opening in long-styled and short-styled types of *Cinchona Calisaya*. Each graph based on observations of 100 flowers. Flowers in the large bud stage were tagged at 6 P.M. or at 6 A.M. for observation.

Breeding

The object of breeding *Cinchona* was to obtain hybrids of good growth, higher quinine content, and disease resistance. Assays of the quinine content of many clones growing in Guatemala had already been made previous to 1944. From these clones, ledgers, ledger hybrids, and hybrids containing 9.5 to 16.1 per cent quinine sulfate in the bark, calisayas containing 7 to 9 per cent, and succirubras containing 5 to 6.7 per cent were selected for breeding. In addition to quinine content, some data on "graftability," rate of growth, thickness of bark, and disease resistance of different clones were available. All of these factors were considered in selecting clones to be used in breeding.

It is not definitely known whether *Cinchona* flowers are wind- or insect-pollinated. For long-styled plants there is ample opportunity for wind-pollinations to be effective in fertilization. However, many insects visit the *Cinchona* flowers. Fifty-three different species of these insects were collected and identified by E. J. Hambleton, entomologist of the U.S.D.A., while on a brief visit to Finca El Naranjo. Many of these insects may be responsible for hybridization pollinations in the field.

TABLE I

TIME REQUIRED TO COMPLETE THE VARIOUS STAGES OF ANTHESIS.*
FIFTY FLOWERS TAGGED ON EACH TREE

Species	Time of tagging buds	Clone	Tree No.	Average duration (hours) for stages in anthesis							
				1	2	3	4	5	6	7	8
C. Ledgeriana	3 P.M.	Short-styled (H1256)	A	7.0	2.5	2.2	28.4	5.0	2.5	12.5	3.0
			B	6.1	0.3	1.7	26.8	10.3	3.5	11.8	3.0
			C	6.8	1.3	1.6	25.4	9.0	6.3	6.8	3.0
			Av.	6.6	1.4	1.8	26.9	8.1	4.1	10.4	3.0
		Long-styled (H1222)	A	9.1	1.2	1.0	28.2	5.0	14.8	12.5	3.0
			B	9.7	1.0	2.6	26.8	4.3	10.5	15.7	3.0
			C	9.2	0.1	1.9	25.6	13.7	8.0	12.5	3.0
			Av.	9.3	0.8	1.8	26.9	7.7	11.1	13.6	3.0
	3. A.M.	Short-styled (H1256)	A	7.2	4.0	3.0	21.8	1.7	3.6	17.5	3.0
			B	9.5	2.8	3.3	22.2	2.1	2.8	18.1	3.0
			Av.	8.6	3.4	3.2	22.0	1.9	3.2	17.8	3.0
		Long-styled (H1222)	A	12.9	1.0	2.9	20.2	2.8	3.7	26.0	3.0
			B	10.7	1.3	2.8	20.9	1.9	3.7	26.6	3.0
			Av.	11.8	1.2	2.9	20.5	2.4	3.7	26.3	3.0
C. Calisaya	6 P.M.	Short-styled	A	11.2	0.3	1.0	21.7	2.5	5.4	16.6	3.0
			B	9.5	0.2	1.8	23.0	2.3	3.9	9.7	3.0
			Av.	10.4	0.3	1.4	22.4	2.4	4.7	13.2	3.0
		Long-styled	A	5.2	0.5	1.4	35.5	4.1	20.3	2.3	3.0
			B	4.4	1.0	1.4	35.4	5.0	19.5	1.0	3.0
			Av.	4.8	0.8	1.4	35.5	4.6	19.9	1.7	3.0
	6 A.M.	Short-styled	A	11.5	1.1	1.7	20.1	2.3	2.0	12.7	3.0
			B	10.3	0.8	2.5	20.8	2.5	2.3	16.4	3.0
			Av.	10.9	1.0	2.1	20.5	2.4	2.2	14.6	3.0
		Long-styled	A	13.9	1.4	1.9	34.7	2.4	15.6	2.9	3.0
			B	13.4	1.7	1.8	33.1	2.8	17.6	3.4	3.0
			Av.	13.7	1.6	1.9	33.9	2.6	16.6	3.2	3.0
C. succirubra	6 P.M.	Short-styled	A	12.7	1.3	0.3	0.3	3.2	11.8	14.0	3.0
		Long-styled	A	13.7	0.8	0.6	1.9	7.3	10.1	13.6	3.0
	6 A.M.	Short-styled	A	13.1	0.8	0.4	0.7	7.0	10.3	15.9	3.0
		Long-styled	A	13.6	0.1	0.1	5.7	7.1	13.7	19.0	3.0

* 1, Bud; 2, bud breaking; 3, bud half open; 4, flower fully open; 5, corolla loose; 6, corolla fallen, stigma in good condition; 7, stigma black; 8, style fallen.

There is ample opportunity for experimental crossing of flowers of the various clones of the different species without storing pollen for any considerable length of time, since their flowering periods overlap in Guatemala. Flowering time is from April through August. However, it should be noted that pollen has been stored successfully for one year in darkness at 10° C. at a controlled relative humidity of 35 to 50 per cent (Pfeiffer, 7).

1944. In August and September a few flowers on several clones of *Cinchona* were available for study. Six calisaya clones were used as seed parents and six as pollen parents. All 25 combinations among the calisaya clones produced 92 capsules with 2,954 viable seed. Ten combinations of calisayas with ledger hybrids, succirubras, and succirubra hybrids gave 43 capsules with 1,686 seed. These first pollinations were made to determine the type of technique best adapted to *Cinchona* breeding. The resulting seedlings are not expected to be of any commercial value.

1945. Self-pollination. By 1945, it was possible to start breeding on a larger scale. Experiments were carried out to determine self-fertility of the clones. Feenstra-Sluiter (5) states that Rant reported in 1908 high self-fertility for one tree of *C. Ledgeriana*. However, Feenstra-Sluiter himself tested two trees and found them completely self-sterile.

In the present experiments, 482 flowers on 12 clones of long-styled ledger, ledger hybrids, and hybrids were self-pollinated, but not one seed capsule was obtained. Of the 390 self-pollinations on short-styled clones, six capsules were obtained on one clone (Z51) while all other self-pollinations failed. These capsules were of normal size and contained the average number of seeds. Thirty premature pollinations on one long-styled clone and 62 on two short-styled clones also failed.

One hundred and sixty-six flowers on five long-styled calisaya clones and 260 flowers on five short-styled clones of this species were also selfed. One capsule was obtained on one long-styled clone (N1634).

Thus it is seen that self-sterility was characteristic of the three species studied since a total of 1,752 self-pollinations made on 55 different clones yielded only eight capsules. Clones on which these capsules were formed (Z51 and N1634) should be retested.

Cross-pollination. For the most part legitimate pollinations (long-styled × short-styled and short-styled × long-styled) resulted in capsules filled with viable seeds while illegitimate pollinations (short-styled × short-styled or long-styled × long-styled) failed completely in seed production. However, one capsule with 13 seeds was obtained from an illegitimate pollination, while 143 others of the same type failed.

The capsules and seeds obtained from the 1945 breeding work are shown in Table II. Indicated as zeros are those pollinations which gave no capsule development. The unusually high percentage of zeros may have been due to heavy rains falling shortly after pollinations and washing the

TABLE II

SUMMARY OF RESULTS OF LEGITIMATE POLLINATIONS MADE IN 1945

Combination				No. of pollinations	Results of pollinations						
Class*		No. made	No. failed		No. of zeros	No. of capsules			Percentage of capsules	No. of seed	Av. No. seed per capsule
						Broken off	Lost	Matured			
1×1	A×B	105	20	6,000	2,669	577	689	2,065	34.4	60,312	29
	B×A	115	36	7,096	3,768	993	732	1,603	22.6	51,940	32
1×2	A×B	6	1	235	81	8	4	142	60.4	4,062	29
	B×A	18	7	145	96	20	0	29	20.0	925	32
1×3	A×B	13	5	299	51	111	41	96	32.1	2,071	25
	B×A	11	5	257	106	66	27	58	22.6	1,834	31
2×2	A×B	25	10	1,224	431	405	127	261	21.3	7,744	30
	B×A	25	5	1,702	829	133	179	561	33.0	15,770	28
2×1	A×B	4	3	81	31	—	47	3	3.7	100	33
	B×A	4	1	365	178	9	23	155	42.5	4,865	31
2×3	A×B	7	4	253	149	60	26	18	7.1	597	33
	B×A	11	3	289	177	51	38	23	8.0	368	16
3×3	A×B	12	2	1,117	—	—	—	—	—	8,777 mg.†	—
	B×A	12	3	1,662	—	—	—	—	—	4,304 mg.	—
3×1	A×B	—	—	—	—	—	—	—	—	—	—
	B×A	7	3	359	—	—	—	—	—	264 mg.	—
3×2	A×B	4	1	211	—	—	—	—	—	900 mg.	—
	B×A	4	1	70	—	—	—	—	—	225 mg.	—

* 1, Ledger, ledger hybrids and hybrids; 2, calisaya; 3, succirubra; A, long-styled; B, short-styled.

† Approximately 3 seeds = 1 mg.

pollen from the stigmas. Also, it is possible that a cinchona plant is limited in its seed production. The number of capsules broken off or lost before maturity is also recorded. Field breeding is naturally subject to some loss of material, but losses in the present tests were unusually high because of winds, falling trees, fungal infection of the inflorescences, and vandalism.

1946. In 1946 three long- and two short-styled ledger clones, one short-styled ledger hybrid, one long- and four short-styled hybrids, one long- and one short-styled calisaya, one long- and one short-styled succirubra, and two long- and one short-styled succirubra hybrids were added to the list of clones selected for breeding. Twenty-one clones of various classes which were found to be either good seed or pollen parents in 1945 were also used in 1946 for mass production of good commercial seeds.

Two hundred and four combinations among the various classes of

Cinchona were made. By March 1, 1947, a total of 34,956 capsules had been collected from which 283 grams of seeds were obtained. Approximately 3,335 capsules were still in the field at this time.

Seeds and seedlings obtained from breeding experiments. Approximately six months after pollinations were made, the first seeds were collected. All seeds from the 1944 and 1945 pollinations were planted and 44,810 seedlings obtained. These were distributed to different locations in Guatemala and Costa Rica for testing. Many seeds from the 1946 pollinations were not yet mature when the author left Guatemala in March 1947.

A breeding garden established at El Naranjo in 1946, in which outstanding clones numbering 23 ledgers, 9 ledger hybrids, 12 hybrids, 5 succirubras, and 9 succirubra hybrids are represented, should facilitate future breeding work. Also the establishment of five seed gardens at other fincas, each garden in an area completely isolated from all other *Cinchona* plantings, will make commercial seed production much more effective.

Further results of the breeding work done in Guatemala from August, 1944 to January, 1947 will be known only after analyses are made of quinine content of the hybrid seedlings and data obtained as to their vigor and resistance to disease. Besides the possibility of having produced a hybrid which will be superior in performance to any known clone of *Cinchona*, the work presented in this paper has determined flower behavior patterns and self- and cross-fertility in established clones of *Cinchona* which will be of value in future investigations in this field.

Colchicine Treatment of Seeds and Seedlings

From seeds soaked for 6, 12, 18, and 96 hours in 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 per cent colchicine solution, a total of 1,662 seedlings survived and were planted in seed beds.

In addition to seed treatment, normal untreated seedlings were removed from nursery beds and immersed in colchicine solutions. After immersion of growing tips for 2, 6, 12, 18, 24, 36, 42, and 48 hours in the same strength of aqueous colchicine solutions as mentioned above, the seedlings were replanted in the nursery position. Of the 1,450 seedlings treated, 1,135 survived and grew well. Also a total of 300 seedlings were treated by dropping emulsions of colchicine (concentrations as above) on all growing points at three-day intervals for a total of three treatments. All of these seedlings survived. Many treated seedlings as well as seedlings produced from treated seeds showed characteristics typical of polyploid plants. Dawson (3) has reported the production of polyploid cinchona plants by the use of colchicine.

All colchicine-treated plants will be carried to field position where they may be observed during their development. It is possible that polyploid plants may have higher alkaloid content than diploids.

Variegated Seedlings

Several green and white variegated seedlings were observed in seed beds of succirubra. The writer does not know of any previous record of *Cinchona* seedlings of this type. They were usually found among plants obtained from seed of succirubra collected from trees of Finca San Pablo. All of the 42 variegated seedlings appeared to be sectoral chimeras. They were removed from the seed bed and placed in nursery beds for further observation.

SUMMARY

Results of investigations of flowering habits and fertility of *Cinchona Ledgeriana*, *C. Calisaya*, and *C. succirubra* conducted in Guatemala from August, 1944 to March, 1947 are described.

Flowers of *Cinchona* are heterostylous, i.e. those of any one clone are either long- or short-styled. No mixtures of intermediate types were observed.

Flower behavior observations were made on a total of 1,097 flowers of the three species. Most *C. Ledgeriana* flowers open at night, between 9 P.M. and midnight. Long-styled flowers of *C. Calisaya* also open at night, but the short-styled clones of this species belong to two types—day opening and night opening.

On the short-styled plant of *C. succirubra* only 15 of the 100 buds tagged opened at all, the remainder of the corollas remained closed and fell from the plant in this condition. A few more of the buds on long-styled trees opened. Hybridization pollinations showed that the stigmas of the succirubra flowers were receptive though the flowers never opened.

Self-sterility was found to be characteristic of the three species since a total of 1,752 pollinations made on 55 different clones yielded only eight capsules.

For the most part, legitimate cross-pollinations, i.e. long-styled × short-styled and short-styled × long-styled, resulted in capsules filled with viable seeds, while illegitimate pollinations, i.e. short-styled × short-styled or long-styled × long-styled, failed in seed production. A summary of the 1945 breeding results, including the classes and number of combinations, the number of failures, matured capsules, etc., is shown in tabular form.

A total of 44,810 seedlings from the seeds produced from the 1944 and 1945 hybridization pollinations as well as the seeds obtained in 1946 were distributed and planted on various fincas in Guatemala and Costa Rica.

Young plants from seeds and seedlings treated with colchicine exhibited characteristics of polyploids.

Several green and white variegated seedlings were observed in seed beds of succirubra.

ACKNOWLEDGMENTS

The writer is indebted to Merck & Co., Inc., for its financial support of the project; to R. P. Lukens, Vice President for Production of Merck & Co., Inc., for his cooperation in the analyses of many bark samples for the determination of quinine content; to B. A. Krukoff, Director of Research of Experimental Plantations for his guidance and criticism during the entire project; to A. E. Bianchi, Central American Manager of Experimental Plantations without whose constant support the work would never have been accomplished; to Owen Smith of Finca Panama, Walter Lind, Finca Moca, Enrique Groenwold, Finca Nueva California, T. R. Jessup, Finca Ona, Jorge M. Benitez, Finca Los Ensayos in Costa Rica, Enrique Hoehn, Finca San Isidro, and P. O. Davies, Finca Helvetia for their hospitality and cooperation; and to two very capable Guatemalan assistants, Carmen Rademann and Victor López.

LITERATURE CITED

1. Cinchona station in Guatemala. Exp. Sta. Rec. **92**: 752. 1945.
2. DARWIN, CHARLES. Different forms of flowers on plants of the same species. 352 pp. D. Appleton & Co., New York. 1877.
3. DAWSON, RAY F. Cinchona polyploids. Lloydia **11**: 81–85. 1948.
4. ENGELBEEN, M. Contribution expérimentale à l'étude de la biologie florale de *Cinchona ledgeriana* Moens. 140 pp. Inst. Nat. pour l'Étude Agron. du Congo Belge. Pub. Sér. Sci. No. 40. 1949.
5. FEENSTRA-SLUITER, C. Waarnemingen en beschouwingen over bloei, bevruchtung en zaadvorming bij *Cinchona Ledgeriana* Moens. 35 pp. Mededeel. Kina-Pröfstation. (Batavia) No. 6. 1919.
6. MORRISON, B. Y. Quinine from seed. Agriculture in the Americas **3**: 131–133. 1943.
7. PFEIFFER, NORMA E. Prolonging the life of *Cinchona* pollen by storage under controlled conditions of temperature and humidity. Contrib. Boyce Thompson Inst. **13**: 281–293. 1944.

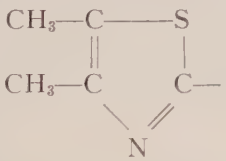
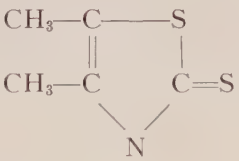
COMPARISON OF A SERIES OF DERIVATIVES OF 4,5-DIMETHYL-2-MERCAPTOTHIAZOLE FOR FUNGICIDAL EFFICACY

C. G. SCHMITT[1]

INTRODUCTION

A series of 4,5-dimethyl thiazoles prepared by Stewart and Mathes (11) offered an opportunity to obtain some information relative to the influence of chemical structure on fungicidal activity. The study was made in the hope that some information might be gained which would point the way toward synthesis of better fungicides. The fungicidal action of heterocyclic sulfur compounds has been investigated by others. Attention was probably first focused upon the thiazoles as fungicides by Alvord (1). Of this group of compounds, mercaptobenzothiazole has received the most attention and is a rather effective general fungicide. It has been used in control of kernel smut of sorghum and bunt of wheat; as an eradicant control for carnation rust; to prevent mildew on leather and textiles; as a remedy for "athlete's foot" and other mycoses and against a number of fungi in agar plate tests (2, 3, 4, 5, 6, 8, 9, 12).

Compounds used were derivatives of two nuclei designated as *R* and *R'* where

R is  and R′ is 

4,5-Dimethyl-2-thiobenzoylthiazole, $RSCOC_6H_5$
4,5-Dimethyl(2-thio-*n*-butyl)thiazole, $RS(CH_2)_3CH_3$
4,5-Dimethyl-2-thioacetonylthiazole, $RSCH_2COCH_3$
o-Nitrophenyl-4,5-dimethylthiazole, $RSSC_6H_4$-*o*-NO_2
Phthaloyl bis-(4,5-dimethylthiazole-2-sulfide), $(RSCO)_2$-1,2-C_6H_4
4,5-Dimethyl-2-thiobenzylthiazole, $RSCH_2C_6H_5$
4,5-Dimethyl-2-(1-thio-4-chlorobutane)thiazole, $RS(CH_2)_4Cl$
N-Diamyl-4,5-dimethylthiazole sulfenamide, $RSN(C_5H_{11})_2$
4,5-Dimethyl-2-(thio-2-amino-ethyl)thiazole, $RS(CH_2)_2NH_2$
Zinc salt of 4,5-dimethyl-2-mercaptothiazole, $(RS)_2Zn$
β-(4,5-Dimethyl-2-thiazolyl)mercaptopropionic acid, $RS(CH_2)_2COOH$
Mg salt of 4,5-dimethyl-2-mercaptothiazole, $(RS)_2Mg$

[1] The B. F. Goodrich Biochemical Laboratory at Boyce Thompson Institute for Plant Research, Inc., Yonkers 3, New York.

4,5-Dimethyl-2-(β-cyanothioethyl)thiazole, $R'(CH_2)_2CN$
4,5-Dimethyl-2-thiomethylcarbamylthiazole, $RSCH_2CONH_2$
Lead salt of 4,5-dimethyl-2-mercaptothiazole, $(RS)_2Pb$
4,5-Dimethyl-2-mercaptothiazole, RSH
Copper salt of 4,5-dimethyl-2-mercaptothiazole, $(RS)_2Cu$
4,5-Dimethyl-2-thio-2,3-thiazoline-3-acetic acid, $R'CH_2COOH$
4,5-Dimethyl-2-thio(2,4-dinitrophenyl)thiazole, RSC_6H_3-2,4-$(NO_2)_2$
4,5-Dimethylthiazole-2-sulfonic acid, RSO_3H

PROCEDURE

The compounds were used at a concentration of 0.001 M in malt extract agar to facilitate direct comparison of activity. Compounds soluble in acetone were used in that solvent and those which would not form a solution in it were ground in a mortar with enough Tween 20 to form a suspension in water. Quantities of chemicals used were weighed accurately to four places on an analytical balance. All chemicals were added to the Difco malt extract agar (final pH 4.6) before sterilization of the medium at 15 pounds for 20 minutes. An untreated lot of agar was reserved for control plates. Fifteen ml. of medium were added to each of the Petri dishes. Following seeding the medium with fungus mycelium and/or spores grown on potato dextrose agar, the plates were incubated at about 21° C. for six days. Colony diameters were determined by averaging two measurements made at right angles to each other on each of the four replicates. The fungi used in the test were *Fusarium lycopersici* Sacc., *Sclerotinia fructicola* (Wint.) Rehm., *Polyporus tulipiferus* (Schw.) Overh., *Phytophthora cinnamomi* Rands, and *Trichophyton rosaceum* Sab. These species were selected because they are economically important and represent types both resistant and susceptible to toxicants.

In addition to inhibition of fungus mycelium in culture, tests were made on prevention of deterioration of cotton fabric. Quadruplicate strips of cotton duck were immersed in 1 per cent preparations of the chemicals, air-dried, and buried in moist humus at 80° F. for two weeks. Strength of the strips following the burial were determined on the Scott tensilometer.

RESULTS AND DISCUSSION

Results of agar plate and soil burial tests are shown in Table I.

The benzoyl, *n*-butyl, and acetonyl derivatives were the most effective compounds in agar plate tests. It is of interest that two of these effective materials possess carbonyl groups.

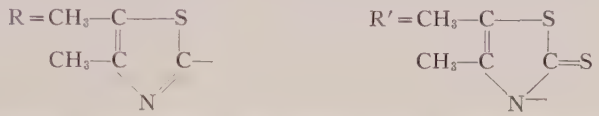
Of the fungi used, *Fusarium lycopersici* and *Polyporus tulipiferus* showed greater tolerance towards this group of compounds than did the other three species. *Phytophthora cinnamomi* and *Sclerotinia fructicola* were intolerant toward most of the compounds tried.

The sulfonic acid and 2,4-dinitrophenyl derivatives were the least inhibitory of the series.

No definite correlation appeared to exist between fungicidal activity and either melting point, boiling point, or molecular weight.

TABLE I

RELATIVE EFFECTIVENESS OF 4,5-DIMETHYL-2-MERCAPTOTHIAZOLE DERIVATIVES AT 0.001 MOLAR AS INHIBITORS OF 5 SPECIES OF FUNGI AND AS PROTECTANTS FOR COTTON FABRIC



Derivative	Colony diameters in mm., average of 4 replications						Tensilometer reading on cotton fabric buried 2 weeks in moist humus at 80° F.
	Fusarium lycopersici	*Sclerotinia fructicola*	*Trichophyton rosaceum*	*Phytophthora cinnamomi*	*Polyporus tulipiferus*	Av. per cent inhibition	
$RSCOC_6H_5$	0	2	0	0	0	99.3	45
$RS(CH_2)_3CH_3$	6	0	0	0	0	97.7	0
$RSCH_2COCH_3$	4	0	1	0	6	95.1	39
$RSSC_6H_4$-*o*-NO_2	19	0	1	0	9	88.2	45
$(RSCO)_2$-1,2-C_6H_4	18	2	3	0	8	85.6	42
$RSCH_2C_6H_5$	14	0	2	0	25	85.0	54
$RS(CH_2)_4Cl$	21	0	4	0	23	78.8	19
$RSN(C_5H_{11})_2$	20	0	4	0	27	78.4	2
$RS(CH_2)_2NH_2$	19	3	8	3	17	74.3	54
$(RS)_2Zn$	31	0	5	6	13	74.3	73
$RS(CH_2)_2COOH$	16	1	7	0	38	73.2	16
$(RS)_2Mg$	38	3	9	10	16	62.9	64
$R'(CH_2)_2CN$	17	2	11	1	46	62.5	0
$RSCH_2CONH_2$	31	7	7	1	52	58.8	0
$(RS)_2Pb$	14	11	2	42	45	48.3	108
RSH	35	3	8	0	17	50.2	—
$(RS)_2Cu$	45	30	13	10	29	48.6	3
$R'CH_2COOH$	31	11	11	27	48	42.5	0
RSC_6H_3-2,4-$(NO_2)_2$	34	27	14	0	52	40.0	0
RSO_3H	39	42	14	0	75	24.0	13
Check	46	45	15	56	70	0.0	0
Check (unburied)	—	—	—	—	—	—	153
LSD 1%	3.6	13.2	0.8	4.2	7.3	31.5*	35.8

* Based upon treatment-species interaction.

The lead salt gave better protection to cotton fabric than did any of the others. The Zn and Mg salts were next in order. The Cu salt was ineffective here as it was in the agar plate tests. The combination in which copper exists is of considerable importance for fungicides in contact with soil. Copper-8-hydroxyquinoline is effective. It works in two ways. In addition

to the direct toxicity of the copper ions, 8-hydroxyquinoline acts as a metal binder which makes required metals unavailable to the fungi (7).

Copper naphthenate is another effective compound. In addition to the action of copper ions, the naphthenic acid part of the molecule displays good fungicidal power (10) which may persist for a considerable period after the copper has been leached away. The residual action of these thiazoles is not great, nor are they capable of forming covalent metal complexes.

The benzyl, benzoyl and acetonyl derivatives, although not so effective in fabric proofing as some of the metal salts, gave fair protection to the fabrics and may be regarded as the most consistent general fungicides in the group. The *n*-butyl derivative was effective in the agar plate test but was ineffective as a fabric preservative.

A glance at the data in Table I reveals that the activity of a given chemical is dependent upon the species involved. The parent compound of the series completely inhibited *Phytophthora cinnamomi*, yet was only slightly inhibitory toward *Fusarium lycopersici*. Specificity has important practical implications. Adequate laboratory testing against the species upon which it is proposed to use a chemical in the field may obviate many of the pitfalls encountered in field testing of new fungicides.

It should be noted that these materials are not so effective as most of the commercial fungicides in current use. Many of the proprietary fungicides are effective at 50 p.p.m. or less. The compounds under consideration here were used at concentrations of 200 to 400 p.p.m. and against some species a few of the derivatives were worthless at these high dosages.

SUMMARY

Of 20 derivatives of 4,5-dimethyl-2-mercaptothiazole tested, the most fungitoxic to five representative species of fungi in agar plate tests were the benzoyl, *n*-butyl, and acetonyl derivatives. In soil burial tests, however, the lead, zinc, and magnesium salts were superior to the non-metallic derivatives and to the copper salt.

LITERATURE CITED

1. ALVORD, EARL B. Fungicides and bactericides. U. S. Patent No. 1,962,109. June 5, 1934.
2. Compte rendu sommaire des travaux poursuivis dans les stations et laboratoires de pathologie végétale. Ann. Epiphyties, N.S. **9**: 271–283. 1943.
3. DAVIES, W. H., and W. A. SEXTON. Chemical constitution and fungistatic action of organic sulphur compounds. Biochem. Jour. **40**: 331–334. 1946.
4. EVERITT, EDWARD L., and M. X. SULLIVAN. The fungistatic and fungicidal action of certain organic sulphur compounds. Jour. Washington Acad. Sci. **30**: 125–131. 1940.
5. FARGHER, R. G. The incidence and control of mould and bacterial attack on textiles. Jour. Soc. Dyers. **61**: 118–122. 1945. (*Abstr. in* Rev. App. Mycol. **25**: 131–132. 1946).
6. HOPLEY, M., and J. R. F. JACKSON. Proofing textiles against rot, mould and mildew. Text. Rec. **63**: 41–42, 59. 1945. (*Abstr. in* Rev. App. Mycol. **24**: 427–428. 1945).

7. ILLMAN, W. I., G. SEMENIUK, A. C. NEISH, and G. A. LEDINGHAM. Rot resistance of cotton duck treated with chelate copper compounds. Canadian Jour. Res. Sec. F. **26**: 311–317. 1948.
8. LEUKEL, R. W. New fungicides and reduced fungicide dosages for the control of kernel smut of sorghum. Phytopath. **32**: 1091–1093. 1942.
9. LOLLAR, R. M. Report on a study and the development of a mould-resistant treatment for leather. Report on mould-resistant treatments for leather. Jour. Amer. Leath. Chem. Assoc. **39**: 12–24; 179–190. 1944. (*Abstr. in* Rev. App. Mycol. **23**: 400–401. 1944.)
10. MARSH, PAUL B., GLENN A. GREATHOUSE, KATHARINA BOLLENBACHER, and MARY L. BUTLER. Copper soaps as rot-proofing agents on fabrics. Indus. & Eng. Chem. **36**: 176–181. 1944.
11. STEWART, FLOYD D., and ROGER A. MATHES. Synthesis of derivatives of 4,5-dimethyl-2-mercaptothiazole. Jour. Organ. Chem. **14**: 1111–1117. 1949.
12. STODDARD, E. M., and J. W. HEUBERGER. Eradicant action of fungicides on spores on living plants. Phytopath. **33**: 1190–1195. 1943.

STUDIES ON THE HERBICIDAL PROPERTIES AND VOLATILITY OF SOME POLYETHYLENE AND POLYPROPYLENE GLYCOL ESTERS OF 2,4-D AND 2,4,5-T[1]

LAWRENCE J. KING AND JOHN A. KRAMER, JR.

INTRODUCTION

The value of the lower aliphatic esters of 2,4-D and 2,4,5-T in weed control is well known. However, these esters are sufficiently volatile to produce injury on unsprayed plants from vapor drift so that their use is necessarily restricted, in many instances by law. Zimmerman and Hitchcock (16) in their paper disclosing the growth-regulating properties of 2,4-D described the ethyl ester and its volatile action. The butyl ester, however, early received a great deal of investigation and also found wide field use. Since vapor from the use of this derivative has resulted in considerable injury to such sensitive crops as cotton and tomatoes its use has been greatly reduced and largely replaced by the amine salts and certain other formulations. Injury has ranged from mild leaf modification to severe stunting and reduction in crop yields.

Polyethylene glycols, principally Carbowax 1500, have been included in many of the early formulations of 2,4-D from their first introduction by Zimmerman and Hitchcock (16) to the more detailed studies by Mitchell and Hamner (6). McNew and Hoffmann (4) have suggested that in the process of heating Carbowax to dissolve 2,4-D some ester formation may have taken place. Polyethylene glycol esters of 2,4-D and 2,4,5-T have been reported by Jones (2), Allen (1), and McNew and Hoffmann (4). Allen alone studied the volatility of these esters.

The present investigations cover a series of polyethylene glycol (PEG) and polypropylene glycol (PPG) monoesters and diesters of 2,4-D and 2,4,5-T which have low vapor pressures. Experimental evidence is presented to show that they perform similarly to other esters in herbicidal action without presenting a hazard to near-by plants. Unlike the currently used esters, these polyethylene glycol esters will not cause any appreciable vapor damage to near-by sensitive plants even if a sprayed and an unsprayed plant are enclosed in the same container (3).

MATERIALS AND METHODS

The polyethylene glycols (9, 13) have the general formula $HOCH_2(CH_2OCH_2)_xCH_2OH$. Those with average molecular weights ranging from

[1] Grateful acknowledgment is given to Dr. A. B. Steele and associates, Union Carbide and Carbon Organic Synthesis Fellowship, Mellon Institute, Pittsburgh, Pennsylvania, for the preparation of the compounds described.

200 to 700 are liquids; those above 1,000 are wax-like solids which have been given the registered trade-mark Carbowax. Either monoesters or diesters may be prepared depending upon the relative quantities of glycol and acid employed in the esterification of 2,4-D. A series of seven 2,4-D and 2,4,5-T polyethylene glycol esters and two polypropylene glycol esters have been studied (Table I). These samples were not highly purified, but

TABLE I

PHYSICAL PROPERTIES OF POLYETHYLENE GLYCOL (PEG) AND POLYPROPYLENE GLYCOL (PPG) ESTERS OF 2,4-D AND 2,4,5-T*

Chemical	Specific gravity, 25/20° C.	Refractive index, 25° C.	Viscosity, 100° F., centi-stokes	Solidification range, ° C.	Melting point, ° C.	Acid equiva-lent, %
PEG 150 2,4-D monoester	1.323	1.5280	222	−28 to −30	—	62.5
PEG 300 2,4-D monoester	1.269	1.5113	203	−20 to −22	—	43.9
PEG 300 2,4,5-T monoester	1.325	1.5197	485	−18 to −20	—	47.5
PEG 200 2,4-D diester	1.365	1.5440	1,032	0 to 5	—	65.0
PEG 200 2,4,5-T diester	—	—	—	—	127–132	75.7
PEG 300 2,4,5-T diester	1.384	1.5440	2,663	20 to 25	—	65.8
PEG 400 2,4,5-T diester	1.363	1.5341	1,754	−2 to 0	—	58.0
PPG 150 2,4,5-T monoester	1.331	1.5260	690	0 to 3	—	65.0
PPG 425 2,4,5-T diester	1.266	1.5170	2,240	0 to 3	—	56.2

* Data supplied by the Organic Synthesis Fellowship, Mellon Institute, Pittsburgh, Pa.

represent samples very close to those produced in commercial development. Concentrations of all chemicals were on an acid equivalent basis.

Numerous tests have been devised for studying the volatility of growth-regulating chemicals. These include the observations following treatment of such hormone responses as curvature of stems and of leaves, and the formative effects in leaves of sensitive plants such as tomato (7, 14, 15, 16, 17), bean (5, 7), and cotton (7, 11), and effects on germination of seeds and the growth of seedlings (8).

The effects on root elongation of seedlings following storage of the dry seed in the presence of chemical vapor may not necessarily be a sure test for growth regulators, but it is known that such compounds affect germination and root growth. Similarly the cucumber root suppression test of Ready and Grant (10) while it, strictly speaking, is not specific for the growth-regulating substances, still offers an accurate measure of comparative differences in toxicity. Herbicidal effectiveness, however, has been

obtained by spray tests on tomato plants and by the oil droplet test on bean plants (12).

In the present experiments the volatility of the PEG esters was studied by observing the responses of non-treated tomato (*Lycopersicon esculentum* Mill. var. Bonny Best) plants enclosed in large glass chambers (45.8 liters) with sprayed plants. Volatility at several temperature ranges was studied by observing the germination and root growth of cucumber seeds (*Cucumis sativus* L. var. Davis' Perfect) after being removed from dry storage in a container with the chemical. Formative effects were evaluated by measuring the modified leaves of cotton (*Gossypium hirsutum* L. var Deltapine 14) plants following exposure to the ester vapors. Herbicidal effectiveness was determined by comparing the polyethylene glycol esters with the butyl ester in the tomato spray test and by the oil droplet test on bean (*Phaseolus vulgaris* L. var. Tendergreen) plants. The comparative toxicity of these two groups of chemicals was determined by the cucumber root suppression test. Field tests for determining herbicidal effectiveness included spraying of Japanese honeysuckle (*Lonicera japonica* Thunb.) and spray treatments designed to control weeds in plantings of sweet and field corn (*Zea mays* L.) at Seabrook Farms, Bridgeton, New Jersey.

LABORATORY RESULTS

In tests designed to detect possible injury from vapor of the polyethylene glycol esters, solutions containing 500 p.p.m. were sprayed on tomato plants which were placed, together with unsprayed plants, in large glass chambers. The butyl esters of 2,4-D and 2,4,5-T were tested in a similar manner. Two unsprayed plants placed in a chamber served as controls. Plant heights were measured at the time of spraying and again 14 days later. The plants were kept in the closed chambers for seven days and thereafter in the greenhouse. The results given in Table II demonstrate a lack of hazard from vapor of the four polyethylene glycol esters, but severe injury leading to death of the plants from the vapor of the butyl esters. In another test similar to the one above solutions of the 2,4,5-T monoester, the triethanolamine salt of 2,4-D, and the butyl ester of 2,4,5-T at the rate of $\frac{2}{3}$ lb. per acre (0.1 mg. per liter of volume of the chamber) were each sprayed on potted tomato plants and placed beside an unsprayed one in the large glass chamber for a period of 72 hours. Cotton seedlings were also included, and after this time interval all of the plants were removed and held in the greenhouse. Figure 1 illustrates these plants four days after the beginning of the test. No epinasty was observed with the PEG ester or the triethanolamine salt, but strong epinasty occurred with the butyl ester. The cotton seedling exposed to the PEG compound and observed three weeks later disclosed no formative effects in the new growth. No effects were observed in the seedling stored with the triethanolamine salt, but the seedling ex-

posed to the vapors of the butyl ester did not survive. Tomato plants similarly exposed to the two PPG esters showed no epinasty or plant injury thus indicating no hazardous effects from vapor would be expected from the use of these two compounds.

In addition to general toxicity certain other effects such as epinasty and structural modifications in the new growth may occur which would not necessarily be reflected in the height measurements. In the first test above

TABLE II

EFFECT OF VAPORS FROM TOMATO PLANTS SPRAYED WITH SEVERAL ESTERS OF 2,4-D AND 2,4,5-T ON THE GROWTH OF UNSPRAYED PLANTS CONFINED IN THE SAME GLASS CHAMBER

Chemical, sprayed at 500 p.p.m.	Average height in cm. after 14 days		Injury rating after 14 days*	
	Sprayed	Unsprayed	Sprayed	Unsprayed
Butyl ester of 2,4-D	1.0	3.5	E	E
PEG 200 2,4-D diester	1.5	8.5	E	A
Control	—	10.2	—	A
Butyl ester of 2,4,5-T	1.0	2.0	E	E
PEG 300 2,4,5-T diester	1.0	8.5	E	A
PEG 200 2,4,5-T diester	1.5	8.7	E	A
Control	—	8.5	—	A
PEG 300 2,4,5-T monoester	0.5	9.0	E	A
Control	—	9.0	—	A
Control	—	8.5	—	A

* A = No injury to E = plant killed.

no epinastic responses were observed in the PEG series, nor were any formative effects observed in the time the plants were under observation. In order to evaluate these formative effects, however, a test has been devised to measure quantitatively such effects in the highly sensitive cotton plant. Staten (11) has shown in a single leaf droplet test that 0.01 ml. of a 1,000 p.p.m. solution of the ammonium salt of 2,4-D was the minimum dosage producing modified foliage; while in spray tests of the entire plant concentrations of 0.0001 to 0.1 p.p.m. did not produce modified growth, but sprays of 1 p.p.m. and higher did so.

In the present tests the effects of vapor alone were studied. Eight cotton seedlings about 22 cm. in height were placed together with a six inch dish containing the chemical (at the rate of 25 mg. of acid per liter volume of the chamber) for a period of 48 hours in the large glass chambers previously described. The room temperature averaged 22.1° C. The plants were then removed and after 14 days height measurements were taken, and after 21 days the leaf measurements were taken. At the time of exposure the smallest recognizable leaf was marked on each plant, and then at 21 days width and length measurements were taken on the second leaf above this

marked one. The butyl ester vapors severely injured the plants resulting in nearly complete cessation of growth. A few plants developed very small modified leaves. The formative effects in cotton leaves from vapors of volatile 2,4-D compounds are quite characteristic and usually result in a very linear or string-like type of leaf. This response lends itself to quantitative expression since the blade length and the blade width measurements

FIGURE 1. Pairs of tomato plants confined in the same chamber for three days after the plant on the right was sprayed with the equivalent of $\frac{2}{3}$ lb. of acid per acre (equal to 0.1 mg. per liter of volume of the chamber). A. Control plants. B. PEG 2,4,5-T monoester. C. Triethanolamine salt of 2,4-D. D. Butyl ester of 2,4,5-T. Photographed after four days.

reflect the extent of the leaf modification. Such data for the above experiment are presented in Table III. The plants exposed to the PEG 2,4-D monoester were not affected in their growth in height, nor were modified leaves produced.

The effects of vapor from several esters upon dry cucumber seeds in storage were also determined. A 100-ml. beaker containing 50 cucumber seeds was sealed in a quart jar alongside a second beaker containing 1 g. of

TABLE III

HEIGHT AND LEAF MEASUREMENTS OF COTTON PLANTS FOLLOWING A 48-HOUR EXPOSURE TO THE VAPORS OF PEG 300 2,4-D MONOESTER AND 2,4-D BUTYL ESTER

Chemical	Concn., mg./l. of vol. of chamber	Leaf epinasty	Av. growth in height of eight plants after 14 days, cm.	Av. measurements in cm. of selected leaf blade after 21 days*	
				Width	Length
PEG 300 2,4-D monoester	25	None	3.6	6.6	5.2
Butyl ester of 2,4-D	25	Extreme	0.8	0.3	0.3
Control	—	None	4.1	6.2	5.6

* Selected leaf was the second one appearing after the smallest recognizable leaf marked on the day of treatment.

the chemical. The seeds had been dried in a vacuum oven to a moisture content of 5 per cent. Seeds were exposed to each of the ten chemicals for seven days at temperatures of 32° F., 70° F., and 90° F. They were then removed to six-inch Petri dishes containing moist filter papers and germinated at 70° F. for five days. Measurement of the roots showed (Table IV) that the vapors of all of the standard esters of 2,4-D and 2,4,5-T were

TABLE IV

CUCUMBER ROOT GROWTH FOLLOWING A 7-DAY EXPOSURE OF THE DRY SEEDS TO VAPORS FROM SEVERAL 2,4-D AND 2,4,5-T COMPOUNDS

Chemical	Av. root length in 5 days of 50 seedlings in mm. after storage of seeds at temperatures indicated		
	32° F.	70° F.	90° F.
PEG 150 2,4-D monoester	21	22	22
PEG 300 2,4-D monoester	23	25	21
PEG 300 2,4,5-T monoester	22	25	25
PEG 200 2,4-D diester	24	23	24
PEG 200 2,4,5-T diester	23	25	20
PEG 300 2,4,5-T diester	24	23	21
PEG 400 2,4,5-T diester	23	23	20
Butyl ester of 2,4-D	18	15	9
Butyl ester of 2,4,5-T	18	12	12
Triethanolamine salt of 2,4-D	20	15	10
Control	23	23	22

injurious at the two higher temperatures, while none of the polyethylene glycol esters caused any injury. There is some indication of root suppression by the butyl esters even at 32° F. These seedlings were not grown to determine whether modification developed in the new growth, therefore it cannot be concluded that the suppression from the toxic ingredient in the vapor is definitely due to the 2,4-D or 2,4,5-T component. In the case of the triethanolamine salt of 2,4-D it should be pointed out that here the

suppression may be due to the excess triethanolamine commonly present in commercial preparations of this material. This would appear to be the logical conclusion since in later tests no formative effects in cotton or epinasty in tomato was observed with this material.

The cucumber root suppression test was also used to compare the activity of polyethylene and polypropylene glycol esters of 2,4-D and 2,4,5-T with the butyl esters. The procedure of Ready and Grant (10) was followed except that the var. Davis' Perfect was used. Tests were replicated twice with 25 seeds in each dish and germinated under conditions described above. Concentrations of 0.01 to 10 p.p.m. were employed. The results appear in Table V. On an acid equivalent basis it can be seen that the

TABLE V

COMPARATIVE EFFECTIVENESS OF SEVERAL ESTERS OF 2,4-D AND 2,4,5-T ON THE GROWTH OF CUCUMBER ROOTS

Chemical	Av. root length after 5 days of 50 seedlings in cm. at various concentrations in p.p.m.				
	10	1.0	0.1	0.01	0.0
Butyl ester of 2,4,5-T	0.6	1.1	1.9	2.4	
PEG 200 2,4,5-T diester	0.7	1.4	2.3	3.0	
					2.5
Butyl ester of 2,4-D	0.7	0.6	1.0	1.2	
PEG 200 2,4-D diester	0.7	0.6	0.7	0.8	
PEG 300 2,4,5-T diester	0.6	1.0	1.4	1.6	
PEG 400 2,4,5-T diester	0.6	1.1	1.5	1.7	
					2.4
PEG 300 2,4,5-T monoester	0.5	0.9	1.5	1.8	
Butyl ester of 2,4,5-T	0.5	0.9	1.3	1.7	
PPG 150 2,4,5-T monoester	0.6	1.1	1.5	1.8	
PPG 425 2,4,5-T diester	0.6	1.1	1.5	1.8	3.3
Butyl ester of 2,4,5-T	0.6	1.2	1.6	1.9	

various PEG and PPG esters of 2,4-D and 2,4,5-T compare favorably with their respective butyl esters for suppressing the root growth of cucumber seedlings.

For comparisons of herbicidal effectiveness the oil droplet test (12) and the tomato spray test were utilized. In the former method 0.01 ml. preparations of the butyl ester of 2,4,5-T and PEG 300 2,4,5-T monoester and the triethanolamine salt were applied on the base of the blade of one of the primary leaves. Four plants were treated with each concentration, and the fresh weights of the top growth above the primary leaves were obtained 10 days later (Table VI). Due to the wide range of weights an analysis of the logarithmic values was made. It is evident that the PEG ester preparations were fully as toxic as the butyl ester.

In the spray test applications of 1, 10, 100, and 1,000 p.p.m. of some PEG and butyl esters of 2,4-D and 2,4,5-T were made to potted tomato

TABLE VI

EFFECTIVENESS OF SEVERAL DERIVATIVES OF 2,4,5-T AND 2,4-D IN SUPPRESSING THE TOP GROWTH OF TENDERGREEN BEANS WHEN APPLIED AS SINGLE DROPLETS TO THE PRIMARY LEAVES

Chemical	Concentration, p.p.m.	Av. top wt. of four plants in mg.
PEG 300 2,4,5-T diester	50	676
	500	62*
PEG 300 2,4,5-T monoester	50	1,768
	500	308*
Butyl ester of 2,4,5-T	50	723
	500	401
Triethanolamine salt of 2,4-D	50	1,332
	500	556
Oil droplet control	—	2,135
Untreated control	—	1,982

* Significantly different from controls. L.S.D. at 5 per cent is 5.5 fold.

plants. Height measurements and injury ratings at 14 and 21 days are given in Table VII. It is evident here, and it was also observed in the test on Japanese honeysuckle, that checking of growth and killing occurs somewhat more slowly with the PEG esters than with the butyl esters. At 21 days the suppressive and killing action of the PEG derivatives is very close to that of the butyl derivatives.

FIELD RESULTS

The diesters of 2,4-D and 2,4,5-T with the polyethylene glycol 200 were tested on Japanese honeysuckle alone and in combination. The materials were dissolved in Solvacide and rendered emulsifiable by addition of 5 per cent emulsifier NPG Tergitol to provide a concentrate with 4 lb. of acid equivalent per gallon. This material was applied at the rate of two quarts in 100 gallons of water per acre to duplicate plots 10×10 ft. of honeysuckle on August 18. Observations made three months later indicated more effective top kill when the mixtures of the two esters containing 2 lb. of acid equivalent in 100 gallons of water were used. Where the two materials were used separately at the same acid equivalent, perhaps about 75 per cent kill of tops occurred in the same interval and no appreciable difference was noted.

The relationship of timing of application to the effectiveness of PEG 200 diester and the triethanolamine salt of 2,4-D was tested in a corn field on the Seabrook Farms near Bridgeton, N. J.[2] Replicated plots, each con-

[2] Acknowledgment is given to Dr. Frank App for making these tests possible.

TABLE VII

EFFECTIVENESS OF SOME POLYETHYLENE GLYCOL AND BUTYL ESTERS OF 2,4-D AND 2,4,5-T IN SUPPRESSING GROWTH OF SPRAYED TOMATO PLANTS

Chemical	Concn., p.p.m.	Growth in cm. after		Injury rating* after	
		14 days	21 days	14 days	21 days
PEG 300 2,4-D monoester	1,000	7.0	7.0	D	E
	100	12.4	14.2	C	D
	10	13.6	15.7	C	C
	1	13.9	16.0	B	B
PEG 300 2,4,5-T monoester	1,000	6.3	6.3	D	E
	100	11.9	12.7	C	D
	10	12.5	14.4	C	C
	1	13.3	15.9	B	B
PEG 300 2,4,5-T diester	1,000	9.1	9.1	D	E
	100	11.2	12.8	C	D
	10	13.1	15.1	C	C
	1	16.8	21.7	B	B
Butyl ester of 2,4-D	1,000	5.0	5.0	E	E
	100	9.2	11.0	C	D
	10	13.2	14.5	C	C
	1	13.4	16.0	B	B
Butyl ester of 2,4,5-T	1,000	9.0	9.0	E	E
	100	9.5	11.5	C	D
	10	12.2	14.6	C	C
	1	13.2	15.5	B	B
Control	—	23.2	27.5	A	A

* A = no injury to E = plant killed.

sisting of 6 rows 200 feet long, were marked out in the fields of sweet corn (var. Golden Cross) and dent corn (U. S. No. 13 hybrid) and sprayed with power equipment at the rate of 1 lb. of acid in 40 gal. of water at the time of seeding or after four and seven days. In addition, each material was applied at the rate of 0.5 lb. to plots in the seven-day application. Weed counts were made 28 days after planting by counting the number of broad-leaved and grass weeds within a 6-inch square over 24 randomized sections of each plot. The weeds were principally crab grass (*Digitaria* spp.), lamb's quarters (*Chenopodium* sp.) and redroot (*Amaranthus* sp.). The data given in Table VIII show that the PEG 200 diester of 2,4-D was similar to the amine salt of 2,4-D in the control of broad-leaved weeds. When the applications were made seven days after planting, somewhat better control of the grass weeds was obtained with the PEG 200 diester. The control of grasses was better when the plots were treated seven days after plantings. An application of the amine salt of 2,4-D at the rate of 1 lb. per acre seven days after planting induced the typical onion-leaf effect on corn as also did the diester, though to a somewhat lesser degree.

TABLE VIII

EFFECT OF THE TIME OF SPRAY APPLICATIONS WITH THE PEG 200 2,4-D DIESTER AND THE TRIETHANOLAMINE SALT OF 2,4-D ON WEED CONTROL IN FIELD AND SWEET CORN PLOTS AT SEABROOK FARMS

Chemical	Rate in lb. per acre	Days after planting when applied	Av. no. of weeds per sq. ft.			
			Field corn		Sweet corn	
			Broad-leaved	Grass	Broad-leaved	Grass
2,4-D amine salt	1.0	0	4.2	4.3	3.2	2.5
PEG 200 2,4-D diester			4.5	2.3	2.8	2.3
2,4-D amine salt			0.3	0.5	1.2	1.5
PEG 200 2,4-D diester	1.0	4	0.0	0.5	0.0	0.8
PEG 200 2,4-D diester	1.0	7	0.8	0.8	0.7	2.7
	0.5		1.5	1.2	2.7	3.0
2,4-D amine salt	1.0	7	2.5	2.3	3.8	2.8
	0.5		3.2	2.8	3.5	3.8
Control	—	—	20.5	9.8	25.6	7.4

SUMMARY

A survey of the volatility and the herbicidal action of some polyethylene and polypropylene glycol esters of 2,4-D and 2,4,5-T has been presented. Esters prepared from polyethylene glycols 150 to 400 and of polypropylene glycols 150 and 425, have been included and the appropriate physical characters of these new compounds have been listed.

The effects of the vapors of certain PEG esters and the butyl esters of 2,4-D and 2,4,5-T were studied on unsprayed tomato and cotton plants enclosed in chambers with plants sprayed with these esters. No marked toxicity to the unsprayed plants was observed with the PEG esters, but severe epinasty resulting in death occurred with the butyl esters.

Dry cucumber seeds were not injured by storage with the various PEG esters for seven days at 32°, 70°, or 90° F. However, the butyl esters of 2,4-D and 2,4,5-T were appreciably injurious at all three temperatures.

In the cucumber root suppression tests for comparative toxicity, the various PEG and PPG esters of 2,4-D and 2,4,5-T compare favorably with the respective butyl esters.

The oil droplet test of the PEG 300 2,4,5-T monoester and diester, the butyl ester of 2,4,5-T, and the triethanolamine salt of 2,4-D on bean plants, has indicated the marked toxicity of the former three compounds. The amine salt tested in aqueous solution was not markedly toxic when tested in this way.

Spray tests on tomato plants for herbicidal effectiveness employing a series of concentrations ranging from 1 to 1,000 p.p.m. have shown the

PEG 300 monoesters and diesters of 2,4-D and 2,4,5-T to be very close in toxicity to the respective butyl esters.

Field tests of the PEG 200 diester of 2,4-D and the PEG 300 diester of 2,4,5-T for control of Japanese honeysuckle were made with sprays applied singly and in combination. When used in combination at 2 lb. per acre, more effective top kill resulted than with the individual esters.

In tests for weed control in field and sweet corn the PEG 200 diester of 2,4-D was compared with the triethanolamine salt of 2,4-D in applications at 0, 4, and 7 days following planting. Data on the weed population 28 days later indicated that the diester was similar in performance to the amine salt when applied at 0 and 4 days after planting but when applied 7 days after planting the diester gave slightly better control.

LITERATURE CITED

1. ALLEN, WM. W. Progress report on volatility of some esters of 2,4-D. Third Proc. Southern Weed Conf. Biloxi, Miss., Feb. 1950. Pp. 7–12.
2. JONES, FRANKLIN D. Methods and compositions for killing weeds. U. S. Patent No. 2,390,941. Dec. 11, 1945.
3. KING, L. J., and J. A. KRAMER, JR. Some new 2,4-D polyethylene glycol diesters. Proc. Northeastern Weed Control Conf. New York City, 1951. Pp. 31–34.
4. MCNEW, GEORGE L., and OTTO L. HOFFMANN. The growth-regulant, herbicidal and physical properties of 2,4-D and related compounds. Iowa State Coll. Jour. Sci. **24**: 189–208. 1950.
5. MARTH, PAUL C., and JOHN W. MITCHELL. Comparative volatility of various forms of 2,4-D. Bot. Gaz. **110**: 632–636. 1949.
6. MITCHELL, JOHN W., and CHARLES G. HAMNER. Polyethylene glycols as carriers for growth-regulating substances. Bot. Gaz. **105**: 474–483. 1944.
7. MULLISON, WENDELL R. The volatility of several salts and esters of 2,4-D as determined by the response of tomato, bean and cotton plants. Proc. Amer. Soc. Hort. Sci. **53**: 281–290. 1949.
8. MULLISON, WENDELL R., and RICHARD W. HUMMER. Some effects of the vapor of 2,4-dichlorophenoxyacetic acid derivatives on various field-crop and vegetable seeds. Bot. Gaz. **111**: 77–85. 1949.
9. The physical properties of synthetic organic chemicals. 16 pp. Union Carbide & Carbon Corporation. New York, N. Y. 1950.
10. READY, DANIEL, and VIRGINIA GRANT. A rapid sensitive method for determination of low concentrations of 2,4-dichlorophenoxyacetic acid in aqueous solution. Bot. Gaz. **109**: 39–44. 1947.
11. STATEN, GLEN. Contamination of cotton fields by 2,4-D or hormone-type weed sprays. Agron. Jour. **38**: 536–544. 1946.
12. SWANSON, CARL P. Two methods for the determination of the herbicidal effectiveness of plant growth-regulating substances in oil solution on broadleaf plants. Bot. Gaz. **107**: 560–562. 1946.
13. Synthetic organic chemicals. 12th ed. 120 pp. Carbide and Carbon Chemicals Corporation. New York, N. Y. 1945.
14. TAFURO, A. J., J. D. VAN GELUWE, and L. E. CURTIS. Drift and volatility comparison of an amine salt and ester form of 2,4-D under field conditions. Proc. Northeastern States Weed Control Conf. New York City, 1950. Pp. 31–35.

15. ZIMMERMAN, P. W., and A. E. HITCHCOCK. Experiments with vapors and solutions of growth substances. Contrib. Boyce Thompson Inst. **10**: 481–508. 1939.
16. ———— Substituted phenoxy and benzoic acid growth substances and the relation of structure to physiological activity. Contrib. Boyce Thompson Inst. **12**: 321–343. 1942.
17. ZIMMERMAN, P. W., A. E. HITCHCOCK, and FRANK WILCOXON. Responses of plants to growth substances applied as solutions and vapors. Contrib. Boyce Thompson Inst. **10**: 363–380. 1939.

REDUCTION OF VEGETATIVE GROWTH AND SEED YIELD IN UMBELLIFEROUS PLANTS BY LYGUS OBLINEATUS

FLORENCE FLEMION AND BETTY TRAVIS MACNEAR

It has long been known that many lots of fresh seed of the commercially important species of Umbelliferae have a relatively low germination capacity. Only recently it has been found that the seed which fail to germinate lack viable embryos and that the missing or shriveled embryos are the result of infestation with *Lygus oblineatus* Say (4, 5). The fact that such a long time elapsed before the connection between *Lygus* and low germination in the Umbelliferae was noted indicates the relative inconspicuousness of the relationship between this insect and the Umbelliferae. It also is of interest that *Lygus* seek out the embryos which comprise such a small and obscure portion of the plant exposed to the insects.

In connection with experiments on the effect of *Lygus* on embryolessness some adverse effect on seed yield of dill and parsnip was noted when these insects were caged with plants at or near the flowering period (5). When *Lygus* fed on flowers or ovaries the entire fruit was destroyed but when feeding occurred at a later stage of development, that is, when the endosperm was mature but the embryo still immature, embryoless seed resulted. In regard to other *Lygus* species it has been reported that *L. campestris* L. can also disastrously reduce carrot seed yield (7). The adverse effect of *Lygus* on seed yield and viability in various species other than the Umbelliferae was discussed earlier (5).

Examination of the literature reveals that *Lygus* are rather omnivorous feeders and damage severely a very wide range of plants (10, p. 12). Retarded and abnormal growth has been reported for alfalfa (1, 9, 14, 15), cotton (13), dahlia (2), guayule (11), etc., while *Lygus* injury to vegetative tissues of umbelliferous species has been reported as damage to parsnip plants (2), rusty dead spots and streaks on the leaf stalks of celery (3, 8, 12), and stunting of carrot due to early damage at the base of the young plants by overwintering adults (7).

It is the purpose of this paper to show the adverse effect on growth, destruction of flowers, and reduction in seed yield in dill (*Anethum graveolens* L.) and fennel (*Foeniculum dulce* Mill.) when *Lygus* are caged with these species.

MATERIALS AND METHODS

Potted dill and fennel plants grown from seed in the greenhouse were transferred, just prior to flowering, to insect-free cages out-of-doors as de-

Copyright, 1951, by Boyce Thompson Institute for Plant Research, Inc.

scribed previously (5, p. 301). In order to eliminate any extraneous insects present all plants and cages were sprayed (4, 5) at the time of transfer, first with tetraethyl pyrophosphate and after a few hours with nicotine.

Subsequently, houseflies (*Musca domestica* L.) were kept continuously in all of the cages during the flowering period to serve as pollinators (5). *Lygus*, collected locally, were added to some of the cages and at adequate supply, approximately 25 insects per cage, was maintained until the seed in the control cages had matured.

The effect on vegetative growth of the plants was recorded and photographs were taken at different stages of development. Data were also obtained on the production and destruction of flowers, umbels, and seed.

RESULTS

In the laboratory several small young dill plants about six inches in height were each confined under a bell jar at room temperature with four or five adult *Lygus*. Within a few days these plants were in very poor condition since the terminal growing point and the tips of a number of leaves were so injured that they wilted and died back. The insects were removed on the sixth or seventh day and subsequently the plants recovered to some extent.

The type of vegetative development obtained when *Lygus* were added at the pre-flowering stage and subsequently maintained thereafter is illustrated in Figure 1. These experiments were conducted out-of-doors in insect-free cages. In the case of dill (Fig. 1 A) the photographs were taken when the seed in the control cages were maturing, while with fennel (Fig. 1 B) the control plants had just begun to flower profusely. A close-up of some of the damage appears in Figure 1 D—note swelling along the stem and destruction of the umbel. It is readily seen that considerable damage occurred when plants were infested with *Lygus* since the growth was retarded and the flowers destroyed (Fig. 1 A, right; B, right). Additional information regarding feeding injury appears in Table I.

Data shown in the table on the effect of feeding by *Lygus* on the destruction of umbels, yield of seed, and embryolessness in the seed produced are similar to that previously reported (4, 5). These insects were extremely destructive to ovaries and flowers as only a few umbels developed and no mature seed was obtained. When *Lygus* were added at the post-flowering stage of development many seeds were produced; however, a considerable number, although they appeared normal, were devoid of embryos. Since these worthless embryoless seeds cannot be segregated, the seed yield was not reduced but the percentage of seed capable of germination was considerably lowered. When *Lygus* were present prior to and during the flowering period the seed crop was a complete failure.

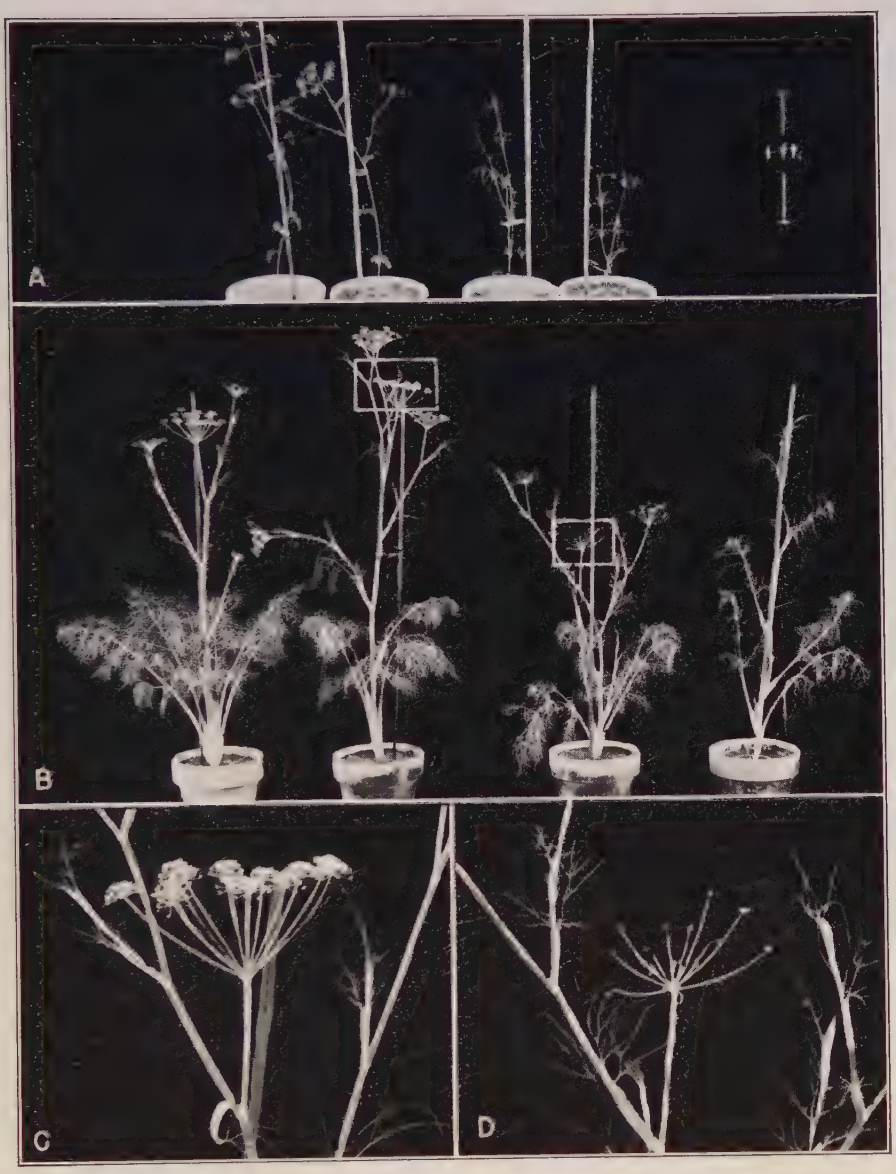


FIGURE 1. Effect of *Lygus* on growing dill (A) and fennel (B and C) plants. A and B: left, control plants; right, plants from cages containing *Lygus*. C and D: enlargement of outlined areas in B, left and right respectively. (A and B, .06 natural size.)

TABLE I

DESTRUCTION OF UMBELS, REDUCTION OF SEED YIELD, AND PRODUCTION OF EMBRYOLESS SEEDS IN THE UMBELLIFERAE BY LYGUS OBLINEATUS

Potted plants caged outside			Effect on seed production				
Species	*Lygus* added*	No.	Total No. of umbels	Seed per plant produced, No.		Embryolessness, %	
				Range	Av.	Range	Av.
Dill	None	7	17	75–354	206	0.0	—
	Before flowering	6	3**	0	—	—	—
	After flowering	7	24	0–338	230	6–91	63.0
Fennel	None	12	47	50–1,594	109	0–4	0.2
	Before flowering	6	0	—	—	—	—

* *Lygus* subsequently maintained in cages until seed maturity.
** All seeds empty.

DISCUSSION

Lygus feed by piercing and sucking. Besides the mechanical injury involved there appears to be a toxic reaction upon the plant cells near the puncture. Results of recent experiments with *Lygus* which had taken in radioactive phosphorus (6) indicate that, while feeding, these insects deposit some oral secretion such as saliva or its equivalent and it is believed that such injection may account, in part, for the toxic effects on various plant tissues.

SUMMARY

Feeding of *Lygus* on dill and fennel plants produced injury to the growing tips of the main stem and branches and also caused destruction of flower buds and umbels. Thus, this insect in addition to being responsible for embryolessness in the Umbelliferae can also adversely affect plant development and seriously reduce seed yield.

LITERATURE CITED

1. CARLSON, JOHN W. *Lygus* bug damage to alfalfa in relation to seed production. Jour. Agric. Res. **61**: 791–815. 1940.
2. CROSBY, C. R., and M. D. LEONARD. The tarnished plant-bug. New York [Cornell] Agric. Exp. Sta. Bull. 346: 461–526. 1914.
3. DAVIS, G. C. Insects injurious to celery. Michigan Agric. Exp. Sta. Bull. 102. 30 pp. 1893.
4. FLEMION, FLORENCE, and JUNE OLSON. *Lygus* bugs in relation to seed production and occurrence of embryoless seeds in various umbelliferous species. Contrib. Boyce Thompson Inst. **16**: 39–46. 1950.
5. FLEMION, FLORENCE, HARRIET POOLE, and JUNE OLSON. Relation of *Lygus* bugs to embryoless seeds in dill. Contrib. Boyce Thompson. Inst **15**: 299–310. 1949.
6. FLEMION, FLORENCE, RICHARD M. WEED, and LAWRENCE P. MILLER. Deposition of P^{32} into host tissue through the oral secretions of *Lygus oblineatus*. Contrib. Boyce Thompson Inst. **16**: 285–294. 1951.

7. HANDFORD, R. H. *Lygus campestris* (L.): a new pest of carrot seed crops. Canadian Ent. **81**: 123–126. 1949.
8. HILL, L. L. Further studies of tarnished plant bug injury to celery. Jour. Econ. Ent. **26**: 148–150. 1933.
9. JEPPSON, LEE R., and G. F. MACLEOD. *Lygus* bug injury and its effect on the growth of alfalfa. Hilgardia **17**: 165–188. 1946.
10. KNIGHT, HARRY H. The plant bugs, or Miridae, of Illinois. Illinois Nat. Hist. Surv. Bull. **22**: 1–234. 1941.
11. ROMNEY, V. E., G. T. YORK, and T. P. CASSIDY. Effect of *Lygus* spp. on seed production and growth of guayule in California. Jour. Econ. Ent. **38**: 45–50. 1945.
12. SHROPSHIRE, L. H., and C. C. COMPTON. Saving garden crops from insect injury. Illinois Agric. Exp. Sta. Circ. 437. 53 pp. 1935.
13. SMITH, GORDON L. California cotton insects. California Agric. Exp. Sta. Bull. 660. 50 pp. 1942.
14. SORENSON, C. J. *Lygus hesperus* Knight and *Lygus elisus* Van Duzee in relation to alfalfa-seed production. Utah Agric. Exp. Sta. Bull. 284, 61 pp. 1939.
15. STITT, LOYD L. Reduction of the vegetative growth of alfalfa by insects. Jour. Econ. Ent. **41**: 739–741. 1948.

DEPOSITION OF P^{32} INTO HOST TISSUE THROUGH THE ORAL SECRETIONS OF LYGUS OBLINEATUS

FLORENCE FLEMION, RICHARD M. WEED, AND LAWRENCE P. MILLER

Many species of Umbelliferae such as carrot, dill and fennel are severely injured by the feeding of *Lygus oblineatus* Say. Although plants are damaged and seed set reduced by feeding on relatively young plants (7), the injury to developing embryos is particularly serious (8, 9). The insects not only reduce seed yield but as high as 50 per cent or more of a fresh crop of apparently well-filled seed are unable to germinate because the embryo is lacking although the endosperm appears normal (10). The injury induced seems to be out of proportion to the extent of feeding, suggesting that some toxic material in addition to the withdrawal of cell fluids may be responsible, as has been proposed by workers with alfalfa (4, 14, 16, 22), bean (3, 20), cotton (6, 17, 19), and other plants (1, 21).

No evidence has been presented, however, to show that toxic materials are injected into the plants during feeding. Studies were undertaken, therefore, to determine whether oral secretions are injected into host tissue. Results reported in this paper have shown, with the aid of radioactive phosphorus,[1] that there is a transfer of material from insect to host during the feeding process.

MATERIALS AND METHODS

Source of insects. The *Lygus* were collected locally and were maintained on green bean pods until used. With one exception all tests were carried out with adult insects.

Supplying P^{32} to the insects. Since *Lygus* are quite small, average weight about 11 milligrams, they would be expected to take in only a small amount of food at any one time. Similarly the quantity of material they would be likely to inject into host tissue would in turn be very small in relation to the food taken in. Further dilution would be involved in that some of the P^{32} would become fixed in the body, a considerable amount would be excreted through the alimentary canal, and the salivary or other secretion that the insect might inject would probably contain a relatively low percentage of phosphorus. It is thus apparent that a rather high radioactivity must be imparted to the insects in order to expect to be able to determine radioactivity in host tissue as a result of feeding.

In the early attempts to obtain insects with sufficient radioactivity to be used in these experiments, small bean (*Phaseolus vulgaris* L., var. Dwarf

[1] The P^{32} used in these experiments was obtained from the Atomic Energy Commission, Oak Ridge, Tenn.

Copyright, 1951, by Boyce Thompson Institute for Plant Research, Inc.

Shell Horticultural) plants were grown in sand cultures in beakers with a a capacity of 400 ml. to which a nutrient medium containing P^{32} as phosphate was added. Subsequently insects were caged with the plants and allowed to feed for several days. Prolonged feeding was impractical because of the short life of the insects. In these experiments with the bean plants, carried out under conditions unfavorable for the survival of *Lygus*, during August and September in the greenhouse, a total of 408 bugs were caged with six plants and 89 were removed alive. The highest radioactivity obtained for any insect was 13,370 counts per minute and for leaf tissue, upon which the insects were feeding, 84,270 counts per minute per square centimeter. As much as one millicurie of P^{32} had been added to an individual culture. Preliminary experiments with these insects, in which they were allowed to feed on immature dill seed, indicated that a higher degree of radioactivity was necessary and that the techniques used in feeding had to be improved in order to avoid contamination of the host tissue by radioactive excrement.

Exploratory attempts to introduce the radioactive phosphorus by external application to and absorption through the dorsal surface exposed after clipping the wing tips or by direct injection into the abdomen were unsuccessful because of the high mortality following these procedures.

Very high radioactivity in *Lygus* was finally obtained by allowing them to feed on a 5 per cent solution of sucrose to which the radioactive phosphorus had been added. Conditions under which this feeding was carried out are illustrated in Figure 1. The sucrose solution was held in a small vial, about 20 mm. in diameter and 20 mm. high, and this vial was placed inside a glass container which in turn was inside two Lucite boxes as shown in Figure 1 B. The glass and Lucite served to confine the radiation so that feeding could be observed without exposure to the radiation. Dental plugs were placed in the sucrose solution in the vial, the vial covered with a plastic screen (lumite mesh 32×32, 0.012 filament) and adhesive tape as shown in Figure 1 A. The insects, which do not normally feed on solutions, were first allowed to feed on sucrose in such vials without P^{32}, and later transferred to vials containing the radioactive phosphorus. The two pieces of adhesive tape were placed very close together (closer than shown in Fig. 1 A) for the feeding on the radioactive solution so that only the mouth parts could penetrate and contamination of the legs of the insect was avoided. Up to two millicuries of P^{32} was added to a vial in order to obtain a high level of radioactivity in the insects.

Subsequent feeding by insects containing P^{32}. Subsequent to feeding on the sucrose solution containing the radioactive phosphate the insects were maintained for a few days on sucrose solution without phosphorus and on bean pods. The reason for a time interval before using the insects for transfer studies was to allow time for the ingested phosphorus to become dis-

tributed throughout the body of the insect and thus be a component of any secretion transferred and to remove any radioactivity remaining on the outside of the mouth parts as a result of mechanical contact with the radioactive feeding solution. Furthermore, the insects did not feed readily

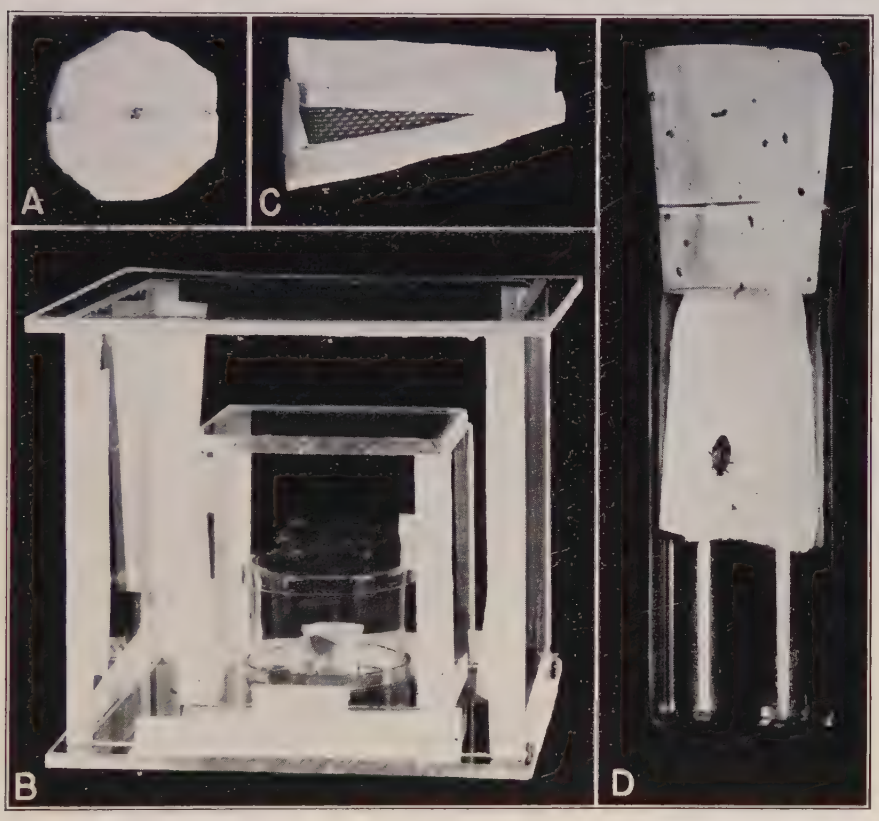


FIGURE 1. Techniques used in transfer experiments. A, Top of glass vial with dental plugs and covered with adhesive tape for feeding on sucrose solution; B, vial in place for feeding on radioactive solution; C, bean pod covered with lumite screen and tape for feeding in interval between feeding on radioactive solution and transfer studies; D, radioactive insect ready to feed on bean pod covered with adhesive tape through which a small hole has been pierced for entrance of mouth parts. (A, C, D, natural size; B, ×0.25)

on bean after having fed on sugar solutions and they were, therefore, not placed on bean pods for the transfer experiments until they had previously fed on bean tissue. In some instances the feeding on sucrose was omitted but the insects were always allowed to feed on the bean pods before use in the experiments.

Sometimes plastic mesh was used on the bean pods as shown in Figure 1 C to more nearly simulate the sucrose feeding. For the feeding in which

the transfer of radioactivity was to be studied the bean pods were covered with adhesive tape with only a small circular opening in the tape through which the mouth parts could penetrate (Fig. 1 D). The insects were kept under observation during the entire period they were confined with bean pods taped in this manner. The length of time the insects fed was noted and if excrement was deposited it could be detected by the slight yellow or pale green color produced. Such excrement was radioactive but the radioactivity did not penetrate through the tape since bean tissue directly under the tape upon which excrement had been deposited was not radioactive. The bean pods were discarded if excrement was noted near the opening through the tape.

The presence of radioactive phosphorus in the bean pods was determined by measuring the radioactivity by counting and through radioautographs. For the counting of both bean pods and insects the material was placed in stainless steel planchets and counts were corrected for a position approximately 18 mm. from a Victoreen mica end-window Geiger-Müller tube. The counts represent about 20 per cent of the disintegrations.

RESULTS

The results obtained with *Lygus* to which radioactivity had been imparted by feeding on sucrose solutions containing P^{32} are summarized in Table I. The insects are naturally short-lived and also sensitive to handling so that there was a high mortality in these experiments. Of 122 insects fed on the radioactive solutions only 52 were removed alive. Of these, 22 subsequently fed on bean tissue in the transfer studies. Fourteen of these fed once, four twice, two three times, and two five times. All pertinent data are summarized in the table. Data on a number of insects have been omitted, either because they deposited excrement near the feeding puncture or they had not taken up sufficient radioactive phosphorus.

The first column in Table I lists the number of counts given by the insects when they were transferred to the bean pods for the observed feeding. Data on the determination of radioactivity of the host tissue are given in the last two columns of the table. The second and third columns show the food supplied to the insects and the length of the interval between the end of the feeding period on the radioactive solution and the transfer experiments. Examination of the table shows that many instances were observed in which the insects imparted radioactivity to the bean tissue. The highest amount of such activity noted was 61 counts per minute; there were ten cases in which the difference between the tissue and background was significant with odds greater than 100 to 1. It will be noted that the amount of radioactivity imparted to the tissue is not necessarily greater with the longer feeding periods. When the same insect was used over a period of several days the amount of radioactivity of the host tissue was often greater

TABLE I

TRANSFER OF RADIOACTIVITY TO BEAN PODS FED UPON BY L. OBLINEATUS CONTAINING P^{32}

Activity of *Lygus*, c.p.m.†	Food supplied before transfer expt., days		Observed feeding on bean pods		Counts per minute above background	
	Sucrose	Bean pods	Days after ingesting P^{32}	Feeding time, minutes	Bean tissue	Required for significance*
613,100	3	1	4	40	28	12**
268,900	1	2	4	3	4	6
			8	50	23	11**
			9	240	61	13**
			10	90	5	8
			11	20	42	9**
188,000	0	4	5	60	28	10**
			6	25	13	7**
			8	50	1	7
162,100	0	5	6	65	1	8
114,000	1	6	8	90	31	12**
108,400	3	1	4	55	41	13**
			5	150	13	12**
103,600	5	1	6	5	−3	—
54,000††	0	3	3	54	13	6**
				70	1	4
			4	26	3	2
				20	1	5
			7	39	−1	—

† Taken when insects were placed on bean pods for transfer experiments. Columns 2 and 3 show how the insects were maintained during the interval between ingestion of P^{32} and use in tests.

†† Nymph when fed P^{32} but adult when transferred to bean; all others adults.

* At the 5 per cent level except when indicated by ** which represents the number required for significance at 1 per cent.

on a given day than for an earlier feeding period. This type of result would indicate that the radioactivity transferred is the result of oral secretions and not merely a mechanical transfer of original radioactive feeding material remaining on the mouth parts. The latter is also improbable in view of the fact that radioactivity was transferred as long as 11 days after the insects had been removed from the radioactive feeding solution.

The insects were under continual observation during the feeding periods in the transfer experiments and it is not believed that any radioactivity was transferred to the bean pods by means of the excrement. In many of the tests given in the table there was no deposition of excrement during the feeding period. The radioactivity of excrement was much higher than

that imparted to the bean pods by the oral secretions. Excrement from the insects used in these tests gave 860 to 6,800 counts per minute, or an activity about 15 to 100 times that of the highest amount noted in the feeding experiments.

Radioautographs were made from a number of the pods which had been found to be radioactive after feeding by *Lygus*. Pods prepared for exposure to the film are illustrated in Figure 2. Immediately below them are positive prints of some of the radioautographs. The tape which had been in place during the feeding period was of course removed before the exposure. Pods which had given counts of 61, 31, and 23 counts per minute above background are shown in Figure 2 A, B, and C, respectively. It is seen that the radioactivity is centered around a very small area. The pod exposed on the right of Figure 2 B had a count of 13 per minute above background. This gave a spot on the negative which is not visible on the print. The other exposures shown are control pods or pods with counts too low to show in the radioautographs.

In the course of these experiments determinations of the radioactivity of insects were frequently made at various time intervals after feeding on the radioactive phosphate and thus data on the rate of reductions of activity are available. It has already been pointed out above that the excrement is highly radioactive so that the decrease in radioactivity of the insects would result from a combination of the loss due to decay plus that lost through the excrement. Data made up by averaging values obtained with a considerable number of individual insects are summarized in Table II.

TABLE II

REDUCTION OF RADIOACTIVITY WITH TIME IN LYGUS WHICH HAD INGESTED RADIOACTIVE PHOSPHATE

Time, days	Per cent of radioactivity remaining		Cumulative loss not accounted for by decay, % (live insects)
	Dead insects	Live insects	
1	93	84	12
3	86	73	18
5	87	57	24
7	72	43	26
14	56	16	27
24	31		
36	17		
57	5		

It is seen that with dead insects radioactivity was reduced to about one-half in 14 days and again another half of the remainder was lost in the next 14 days. This represents the normal decay of the radioactive phosphorus. With live insects the rate of loss was considerably greater. The data in the last column show the cumulative loss up to 14 days resulting from losses in excrement. It is seen that in the first five days about 24 per cent of the in-

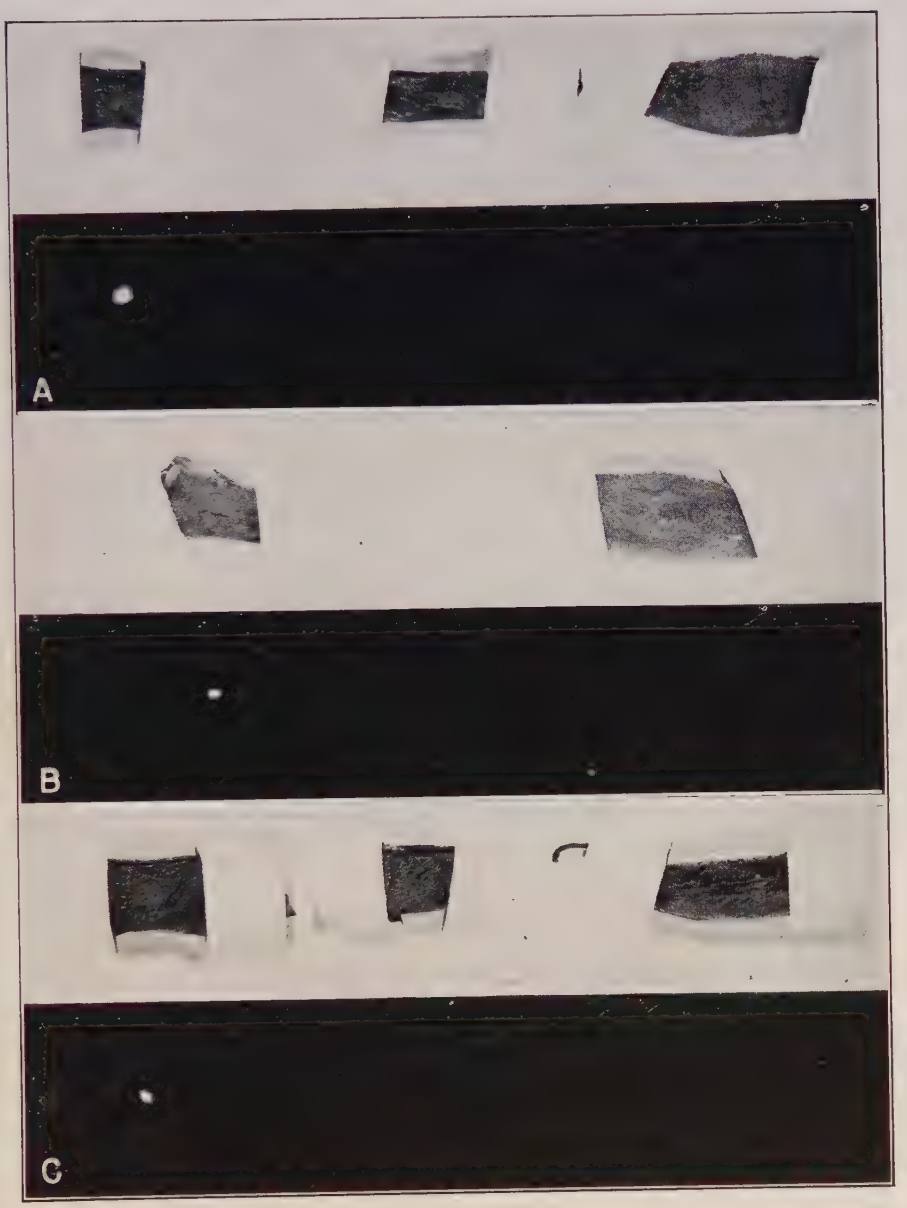


FIGURE 2. Results of exposing film to bean tissue upon which radioactive *Lygus* had fed. The upper rows of A, B, and C show the bean pod tissue prepared for exposure to the film and immediately below positive prints obtained from the corresponding radioautographs are reproduced. The activity of the tissue exposed, in counts per minute above background, left to right, beginning at the top was 61, 1, control; 31, 13; 23, 13, control. (M×0.88)

gested radioactivity is lost in this way and that thereafter most of the reduction in radioactivity results from decay.

DISCUSSION

That some insects inject materials on feeding has been well known for a long time and is especially easy to demonstrate with insects that transmit diseases in which part of the life cycle of the causative agent takes place in the body of the insect. The outbreak of disease in the host is elegant proof of transfer from insect to host. There are also instances in which feeding of insects on animals induces a reaction which would seem to be the result of injection of an irritant material by the insect. Insects which pierce host tissue with their mouth parts in order to withdraw fluids are thought to secrete a lubricant to ease the penetration and/or facilitate the feeding process (2).

Injury caused by *Lygus* has been reported to appear greater than the mere withdrawal of fluids would warrant (4, 16, 20, 22) and the injection of toxic secretions has been postulated (1, 3, 14, 17, 19, 21). It has been noted that the amount of injury is not always directly related to the length of feeding (6, 17), which would support the thesis that secretions play a role. With the use of radioactive tracers it has been possible to establish that there is some transfer of material from insect to host. In these tests, also, the amount of material transferred was not necessarily greater with longer feeding periods. It may be possible to speculate as to the probable amount of such secretions observed in these experiments. If the phosphorus content of the oral secretions were known and data were available on the proportion of the total phosphorus in these insects to be found in the oral secretions present at any one time, it would be possible to estimate fairly accurately the quantity of secretions transferred in the feedings studied in the present experiments. These data are of course not available and only crude approximations are possible.

Some studies of phosphorus distribution in various species of insects and on the content of the salivary glands have been reported. The conclusions reached have differed considerably with different insects. Irwin, Spinks and Arnason (15) have reported that P^{32} accumulates particularly heavily in the salivary glands of the larvae of *Drosophila*. Hahn, Haas, and Wilcox (11), working with mosquitoes (*Aedes aegypti* L.) have dissected out the salivary glands of insects which had fed on glucose solutions containing approximately 30 millicuries of P^{32} and they found the activity of the glands to vary from 334 to 14,000 counts per minute. In studies carried out by Lindsay and Craig (18) on the distribution of radiophosphorus in the wax moth (*Galleria melonella* L.), mealworm (*Tenebrio molitor* L.), cockroach (*Blatella germanica* L.) and the firebrat (*Thermobia domestica* Pack), they found little or none in the salivary glands. No information seems to be available on the phosphorus content of the saliva itself.

In the experiments with *Lygus* a high level of activity in the whole insect was necessary before sufficient radioactivity was transferred on feeding to be detected. Insects having an activity of 100,000 to 600,000 counts per minute imparted a maximum of 30 to 60 counts per minute to tissue fed upon, thus representing a dilution factor of only about 3,000 to 10,000. This is relatively small considering that the salivary secretions no doubt are high in water content and phosphorus probably comprises a relatively small proportion of the total solids present. In man 1 ml. of saliva would represent only from about 1/8,000,000 to 1/3,000,000 of the total phosphorus content (calculated from data in Hawk, Oser, and Summerson, 13).

In experiments carried out by Hamilton (12) intake of fluid and subsequent transfer on further feeding was studied in *Myzus persicae* Sulz. with the use of radium (D+E+F). In these tests, assuming the polonium content of the secretion to be the same as that of the feeding solution originally used to supply it, the insects transmitted an average of 6.9 per cent of the imbibed solution to the leaf. In view of the low penetrating power of the alpha particles given off by polonium, corrections could be made for radioactivity transmitted from the excrement of the feeding insects to the leaf surface. Feeding on the test leaves was carried out immediately following the intake of the radioactive solution and this may account, in part at least, for the transfer of a much larger percentage of the radioactivity as compared with that in the experiments with *Lygus*.

Carter (5) studied secretions of the pineapple mealybug (*Pseudococcus brevipes* Ckl.) using radioactive phosphorus. He found considerable radioactivity in agar upon which radioactive insects had fed and also demonstrated radioactivity in plants after feeding by the insects. No mention is made of any provision for excluding radioactive excretions other than the oral secretions and it is thus not possible to know whether any of the imparted radioactivity came from sources other than the mouth parts.

These experiments with *Lygus*, of course, do not offer any evidence that the oral secretions are toxic. By establishing that secretions are actually involved in the feeding, and that they probably are fairly heavy in comparison to the size of the insects, the results serve as a logical background for further tests with these secretions. It may be possible to trap the secretions in some medium and to test same for any destructive effect on plant tissue.

SUMMARY

Lygus oblineatus were made highly radioactive by allowing them to feed on sucrose solutions to which radioactive phosphate had been added. Such insects on subsequent feeding on bean pods imparted radioactivity to the tissue at the feeding site as shown by counting and by radioautographs. These results thus offer strong evidence that these insects on feeding inject oral secretions into host tissue.

LITERATURE CITED

1. ADDICOTT, F. T., and V. E. ROMNEY. Anatomical effects of *Lygus* injury to guayule. Bot. Gaz. **112**: 133–134. 1950.
2. AWATI, P. R., and JOHN WOLFE-BARRY. The mechanism of suction in the potato capsid bug, *Lygus pabulinus* Linn. Proc. Zool. Soc. [Lond.] **2**: 685–733. 1914.
3. BAKER, KENNETH F., WILLIAM C. SNYDER, and A. H. HOLLAND. *Lygus* bug injury of lima bean in California. Phytopath. **36**: 493–503. 1946.
4. CARLSON, JOHN W. *Lygus* bug damage to alfalfa in relation to seed production. Jour. Agric. Res. **61**: 791–815. 1940.
5. CARTER, WALTER. The oral secretions of the pineapple mealybug. Jour. Econ. Ent. **38**: 335–338. 1945.
6. EWING, K. P. Effects on the cotton plant of the feeding of certain Hemiptera of the family Miridae. Jour. Econ. Ent. **22**: 761–765. 1929.
7. FLEMION, FLORENCE, and BETTY TRAVIS MACNEAR. Reduction of vegetative growth and seed yield in umbelliferous plants by *Lygus oblineatus*. Contrib. Boyce Thompson Inst. **16**: 279–283. 1951.
8. FLEMION, FLORENCE, and JUNE OLSON. *Lygus* bugs in relation to seed production and occurrence of embryoless seeds in various umbelliferous species. Contrib. Boyce-Thompson Inst. **16**: 39–46. 1950.
9. FLEMION, FLORENCE, HARRIET POOLE, and JUNE OLSON. Relation of *Lygus* bugs to embryoless seeds in dill. Contrib. Boyce Thompson Inst. **15**: 299–310. 1949.
10. FLEMION, FLORENCE, and ELIZABETH WATERBURY. Embryoless dill seeds. Contrib. Boyce Thompson Inst. **12**: 157–161. 1941.
11. HAHN, P. F., VICTOR A. HAAS, and AIMEE WILCOX. Arrest of development of *Plasmodium gallinaceum* in mosquitoes (*Aedes aegypti*) by radiation. Science **111**: 657–658. 1950.
12. HAMILTON, M. A. Further experiments on the artificial feeding of *Myzus persicae* (Sulz.). Ann. App. Biol. **22**: 243–258. 1935.
13. HAWK, PHILIP B., BERNARD L. OSER, and WILLIAM H. SUMMERSON. Practical physiological chemistry. 12th ed. 1323 pp. The Blakiston Co., Philadelphia. 1947.
14. HUGHES, JOHN H. The alfalfa plant bug, *Adelphocoris lineolatus* (Goeze) and other Miridae (Hemiptera) in relation to alfalfa-seed production in Minnesota. Minnesota Agric. Exp. Sta. Tech. Bull. 161. 80 pp. 1943.
15. IRWIN, R. W. B., J. W. T. SPINKS, and T. J. ARNASON. Deposition of P^{32} in *Drosophila*. Canadian Jour. Res. Sec. D. **28**: 137–142. 1950.
16. JEPPSON, LEE R., and G. F. MACLEOD. *Lygus* bug injury and its effect on the growth of alfalfa. Hilgardia **17**: 165–182. 1946.
17. KING, W. V., and W. S. COOK. Feeding punctures of mirids and other plant-sucking insects and their effect on cotton. U. S. Dept. Agric. Tech. Bull. 296. 11 pp. 1932.
18. LINDSAY, EDER, and RODERICK CRAIG. The distribuion of radiophosphorus in wax moth, mealworm, cockroach and firebrat. Ann. Ent. Soc. Amer. **35**: 50–56. 1942.
19. MCGREGOR, E. A. *Lygus elisus:* a pest of the cotton regions in Arizona and California. U. S. Dept. Agric. Tech. Bull. 4. 14 pp. 1927.
20. SHULL, WESLEY EARL. An investigation of the *Lygus* species which are pests of beans (Hemiptera, Miridae). Idaho Agric. Exp. Sta. Res. Bull. 11. 42 pp. 1933.
21. SMITH, KENNETH M. Investigation of the nature and cause of the damage to plant tissue resulting from the feeding of capsid bugs. Ann. App. Biol. **7**: 40–55. 1920/1921.
22. SORENSON, C. J. *Lygus hesperus* Knight and *Lygus elisus* Van Duzee in relation to alfalfa seed production. Utah Agric. Exp. Sta. Bull. 284. 61 pp. 1939.

THE ECONOMICS OF USING FUNGICIDES[1]

R. H. Wellman[2]

Fungus diseases of plants cost each man, woman, and child in the United States approximately 10 dollars per month in spite of the best control measures now available. This $1\frac{3}{4}$ billion dollar annual loss is stated in terms of the consumer, because farmers, as businessmen, must add their losses to the selling price of their product if they are to stay in business, and this increase is passed along to the consumer.

Better control of fungi would mean lower food prices for the consumer. If no fungicides were available, this crop loss would jump to over four billion dollars a year. Without fungicides more than a billion dollars worth of such crops as apples, potatoes, grapes, and cherries could not be grown at all in the important producing areas east of the Mississippi. Another billion dollars would be lost annually in reduced yields on such crops as corn, cotton, and wheat. Here seed treatments are used to prevent fungus attack to the seeds or young seedlings. In this case, the fungi rarely wipe out the crop but do reduce the yield so there is less crop per acre.

The billion dollars worth of crops that could not have been grown without fungicides is saved by using chemicals costing 20 million dollars and labor and equipment costing 30 million dollars. In other words, for these crops each nickel spent in controlling disease makes possible the production of crops valued at one dollar.

The return from seed treatment fungicides is in general even larger. It costs less than three cents per acre to treat corn seed. It has been shown that the increased yield per acre is worth as much as four dollars. Here then a single penny spent for preventive treatment returns a dollar. An extreme case is seed treatment of spinach where thirty cents worth of chemical as a seed treatment has been shown to return about three hundred dollars in increased yield.

General statements on the value of fungicides become meaningless when applied to specific crops on a given acre in any one year. Nearly all fungicides are preventive treatments and hence are a form of insurance. As with other forms of insurance, their worth is proven only by averaging all the results obtained with them. A certain farmer may, in a certain year, be applying them needlessly while another farmer may be getting benefits greatly above average. However, since severity of disease cannot be pre-

[1] Invitational paper presented before the American Chemical Society in Philadelphia, Pa., on April 12, 1950.

[2] Head, Biological Research Department, Carbide and Carbon Chemicals Company, Union Carbide and Carbon Corporation.

dicted in advance as it depends on climatic conditions, both farmers must use the fungicides.

The benefits of spraying tomatoes are shown in an unusual form in data published by McNew.[3] These data are presented in Table I.

TABLE I

INCREASED POUNDS OF TOMATOES PER POUND OF COPPER APPLIED
(After McNew, p. 38)

Pounds of copper per acre	Severity of blight epidemic, per cent	Fruit per acre in lb.	Increased fruit per lb. of copper
19.5	40.9	31,116	466
9.8	49.4	30,240	838
4.9	53.0	27,472	1,111
0.0	75.4	22,027	—

Although the greatest increment in pounds of fruit per pound of copper applied occurred with the lowest copper dosage, the greatest yield per acre was obtained with the highest amount of copper used. Since the farmer has already invested a major portion of cost of production in planting and cultivation, he is greatly interested in increasing the yield per acre for this brings his unit cost down and his profit up. For the highest yielding treatment, the cost of fungicide to the farmer was $7.92 per acre. The increased yield over the untreated plot was 9,089 lb. of tomatoes. In 1946, these tomatoes would have been worth $32 a ton or $145 for the total increased yield. In this case, the farmer got back 18 cents worth of tomatoes for each penny invested in fungicide.

Table I shows that there was an increase in tomato yield as the disease index went down. With the material used, the blight index was still 40 at the highest concentration of fungicide. If a rough linear extrapolation is made, it becomes apparent that at a disease index of 30, the yield would have been 34,000 lb. At a disease index of 20, the yield would have been 37,000 lb. provided other factors for growth did not become limiting.

Using the copper fungicide and assuming the more probable logarithmic relationship between concentration of fungicide and disease control, it would take about 45 lb. of fungicide to bring the disease index to 30. This would be 25 lb. more than the highest dosage and would have cost $10.02 more. But this would have produced an additional $1\frac{1}{2}$ tons of tomatoes worth $48. It would have taken 100 lb. more fungicide to reduce the disease index to 20; this would have cost $25 and produced additional tomatoes worth $96.

The economics of these higher doses seem sound providing yields continue to increase as more fungicide is used (of course, the productive capac-

[3] NcNew, George L. The use of fungicides during war. X. Economical use of copper in tomato spraying. The Canner **96**(16): 16, 36–40. March 20, 1943.

ity of the soil will provide an upper limit for such increase). The mechanics of applying such heavy slurries as would have to be handled and their probable phytotoxicity, however, make it impractical. Obviously, a more effective fungicide that would reduce the disease index to 20 would be economical at a considerably greater price per pound than the copper fungicide.

Perhaps the best way to sum up the economics of fungicides to the farmer is to say that he is now using fungicides that cost over 34 million dollars a year. The large bulk of this sale is in copper and sulfur fungicides which together account for some 24 million dollars of sales. However, a new and rapidly growing market is in the new fungicides which are estimated to be enjoying a current volume of over 10 million dollars a year. This market was largely non-existent 10 years ago and it should still be considered as in its infancy.

Since $1\frac{3}{4}$ billion dollars of fungus damage is still uncontrolled, it would seem reasonable that, as fungicides are found that reduce this loss, the market for fungicides can grow at the rate of 1/10 to 1/20 of the dollar value of the losses they obviate. If this loss can be cut to $\frac{3}{4}$ billion dollars, a new market should be created for about 50 million dollars worth of fungicides. Other means of combatting fungi, particularly resistant varieties, are being developed. Experience shows that the war against fungi is by no means static. A new resistant variety is developed and a new strain of the fungus attacks it. The loss from one disease is cut down and that from another increases. As more knowledge is obtained, new fungi are found to cause economically important losses. There are imminent possibilities that efficient soil fungicides and chemotherapeutants will be developed. From the over-all view, therefore, the estimate of 50 million dollars worth of untapped fungicide market would seem conservative.

Eight million dollars of business in new fungicides is now being done, compared to a decade ago. The new fungicides have come on the market for the following reasons:

1. They may allow for greater yield or higher quality through less adverse action on the crop itself.

2. They may have some auxiliary action such as holding insects at a lower level.

3. They are more effective.

Since fungicides are used at different concentrations and may be applied at different frequencies, the farmer is principally interested in the cost per acre per season and not in the cost per pound of fungicide. Even on the basis of cost per acre per season the new fungicides have been more expensive than their predecessors. This is possible because of their additional advantages as listed above.

The advantage of what would seem to be only slightly greater effective-

ness in disease control becomes apparent on consideration of the economics involved. In normal years (there were some exceptions during and immediately following the war) farming is no more profitable than manufacturing. A profit of 15 per cent before taxes is considered favorable. Most of the pre-harvest costs of farm production are fixed even if no crop is harvested. Suppose that with old-line fungicides the farmer is able to control disease to the extent of 95 per cent and a new fungicide makes possible a 99 per cent control. Suppose further that this is directly reflected in increased yield. He has an additional four per cent yield, but since most of his costs are fixed, his profit is increased not by four per cent but by several times this amount. Actually it will be by appproximately the ratio of $19/15 \times 100$ minus the increased cost of the fungicides and increased harvesting and marketing charges of the additional crop. Thus his cost for the new fungicide may well be higher and yet the net effect be considerably more profit.

This discussion has been limited to agricultural fungicides, but similar reasoning applies to the field of wood preservatives and industrial preservatives which together are reducing a potential billion dollar yearly loss.

During the last decade, research in developing new fungicides has greatly increased. This research has borne fruit in the eight million dollar market in new fungicides. However, this is the first crop only and the tree of fungicidal research is not yet in full bearing, nor will it be until over 50 million dollars of new markets are developed.

TESTING TECHNIQUES[1,2]

S. E. A. McCallan

It is probable that as many as 50,000 chemicals have been examined during the past decade as possible fungicides. Naturally the vast majority of these chemicals cannot have been tried under the expensive and time-consuming conditions of use, but rather by various screening and testing techniques. Largely because of this great interest in the search for new fungicides much stimulus has been given to the improvement and development of suitable testing techniques. Some professional organizations, such as the American Phytopathological Society, The American Wood-Preservers' Association and The American Society for Testing Materials, have encouraged the development of standard techniques.

Since fungicides are applied in many and varied fields such as in agriculture for the spraying of plants, the protection of seeds, and the fumigation of soil, in industry for wood and textile preservation, and in medicine, the appropriate testing techniques are also necessarily varied. While a chemical which shows fungicidal effectiveness for one use may be expected to show some degree of effectiveness for another use, the analogy usually is not sufficiently close to be of much practical help. In other words either because fungicides behave very differently, or because we as yet understand so little about the nature of fungicidal action or probably both there is no universal method of testing effectively for all fungicidal properties.

The action of most fungicides is protective, that is, they are applied to protect the uninfected surface from attack by the fungus. In the case of leaves or seeds, a layer of chemical which is toxic to the attacking fungus is placed over the surface. With wood preservation the chemical is allowed to penetrate a certain distance and with textile or plastic preservation the material is commonly permeated with the protective fungicide. In the less common case the attempt is made to disinfect the infected part as in medical application and soil fumigation and a few cases of plant disease control.

CLASSIFICATION OF METHODS

All methods of evaluating fungicides embody two general systems: (a) fungus and chemical and (b) fungus, chemical, and host. Methods dealing

[1] Invitational paper presented before the American Chemical Society in Philadelphia, Pa., on April 12, 1950.

[2] A more detailed account of "Bioassay of Agricultural Fungicides" may be seen in Agr. Chem. 2(9): 31–34, 67; 2(10): 45. 1947, or Boyce Thompson Inst. Prof. Pap. 2(4): 23–33. 1947.

Copyright, 1951, by Boyce Thompson Institute for Plant Research, Inc.

with the fungus and chemical are in general simpler, more rapid, and of greater precision. However, the results obtained are perhaps more academic than practical. These methods are best suited to preliminary screening. On the other hand the methods involving fungus, chemical, and host are more complex, and less precise since the conditions are less well controlled. They are likely to be more time-consuming and expensive. However, the results are more specific and more practical since they are more closely applicable to actual use.

FUNGUS-CHEMICAL TECHNIQUES

In a short introduction, brief mention can be made of only a few of the more important and representative techniques. Among the fungus-chemical techniques there are three main types: first, spores are suspended in solutions or suspensions of the chemical, removed at stated intervals and placed on a suitable medium where the presence or absence of growth is observed. This procedure measures true fungicidal action or killing power and is more commonly used in the medical and bacteriological field. In the second type the chemical is incorporated in a nutrient medium and fungus spores or mycelium added and their growth noted. This method in numerous modified forms is commonly used for screening purposes in the agricultural field and in textile and wood preservation. While simple it has the marked disadvantage that the nutrient medium may react with the chemical and interfere with its fungistatic activity. With the third method spore suspensions are exposed to the chemical on glass slides and the percentage germination recorded. The chemical may be mixed with the spore suspension and aliquot drops pipetted on the slides or the chemical may be sprayed or settled on the slides by more or less elaborate equipment and the spores added later. The method is widely used in the agricultural field and has been standardized by the American Phytopathological Society. A promising chemical should inhibit the germination of half the spores (the ED_{50}) at a concentration of less than 10 p.p.m. The fungi employed in these techniques represent a wide range. In the standardized slide-germination method the ones most commonly used are *Sclerotinia fructicola* (Wint.) Rehm, *Stemphylium* (formerly *Macrosporium*) *sarcinaeforme* (Cav.) Wilts., and *Alternaria oleracea* Milbraith.

FUNGUS-CHEMICAL-HOST TECHNIQUES

The complex fungus-chemical-host system is intended to simulate more or less conditions of use and hence many techniques are employed. Host as used here is defined to include inanimate materials, such as wood blocks and textiles, as well as animate objects.

In the agricultural field we are concerned primarily with evaluating fungicides which will control foliage, seed-borne, and soil-borne diseases.

Various foliage disease methods have been developed which are representative of the more important groups such as late blight of tomato, early blight of tomato, apple scab, several rust diseases, and powdery mildews. The last is really a case of disinfection rather than protection. Commonly, potted plants are sprayed in the laboratory with chemicals in a dosage series, inoculated with the appropriate fungus, incubated, and eventually returned to the greenhouse where disease lesions appear, are counted, and are expressed as a percentage of the control unsprayed plants. Many effective fungicides will give good disease control at a concentration of less than 0.1 per cent by this method. Weathering techniques to simulate rain and sunlight may be introduced but more research is required here. A disadvantage of these foliage disease techniques is variability in response. Seed-borne disease techniques are limited mostly to the cereal smuts. The infested seed is treated with the chemical and planted, but the efficiency of the treatment cannot be determined until maturity when the heads of grain are examined for smut. An exception is cotton anthracnose which requires only a few weeks for results. Screening techniques to evaluate soil fumigants are relatively undeveloped. However, techniques for seed protectants from soil-borne organisms are simple, direct, and fairly well correlated with field results. The seed is treated with the chemical, most commonly in dust form, planted in fungus-infested soil, and the percentage emergence recorded.

In wood preservation methods, the use of impregnated wood blocks is favored. Uniform blocks of wood are impregnated with the chemical under specified conditions, then placed over actively growing cultures of wood-destroying fungi on malt agar. Or, in another procedure, soil is used instead of the agar and the fungus is grown on thin feeder strips of wood previously inoculated and both a control and impregnated block are inserted in each test bottle. At the end of the test which requires several months the blocks are examined for loss of weight and extent of decay. Some of the commonly used fungi are various species of *Fomes*, *Polyporus*, *Poria*, and *Lenzites*.

The evaluation of fungicides to protect textiles, plastics, electronic equipment, etc. utilizes three general types of exposure: known organisms on synthetic media, "tropical chambers," and soil burial. In the first case a strip of the chemically impregnated or coated textile or plastic is placed on a synthetic medium consisting usually only of mineral salts so that the carbon must be furnished by the test object, and inoculated with a single or mixed culture. Fungi commonly used for this purpose are species of *Aspergillus* and *Penicillium*, *Chaetomium globosum* Kunze and *Myrothecium verrucaria* (Alb. & Schw.) Ditm. ex Fr. (formerly known as *Metarrhizium glutinosum* Pope). Tropical chambers with a luxuriant fungus flora artificially or naturally introduced may be used for exposing the objects treated with fungicides. High temperatures and relative humidities are employed in these chambers as in the previous method to give an accelerated or trop-

ical-like test. Soil burial tests are the simplest and probably most drastic of these methods. Here strips of fungicide-treated textile or plastic are buried vertically in a rich top soil. The duration of these various tests is from a week to several months, at the end of which time the specimens are examined for extent of fungus growth, tensile strength, and loss of other physical properties.

Statistical methods. Considerable impetus and understanding in the development and use of techniques for evaluating fungicides have been given by the use of statistical methods. Attention has been focused especially on appropriate experimental designs, the errors of technique, the need for replications, and the differences required for significance. In some of the agricultural techniques in particular, the use of the dosage-response curve has aided in making comparisons and in interpreting the nature of fungicidal action. The data when plotted on logarithmic probability paper will usually but not always give a straight line response. The dose or concentration of chemical required to give a 50 per cent response or 95 per cent response, for example, 95 per cent of the spores inhibited from germination or 95 per cent reduction of disease lesions, is called respectively the ED_{50} or the ED_{95}.

CONCLUSION

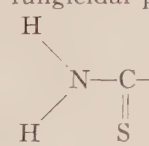
At the present time there is much room for improvement in the methods for evaluating fungicides especially toward better correlation with field results. It is not uncommon to find a chemical with high fungicidal value, but it is difficult to find one that also has no deleterious effect upon the host, or upon humans, is stable, withstands weathering, is economical, and is better than the available fungicides. For every 1,000 chemicals screened as fungicides probably less than one would be expected to be a successful practical fungicide. It is to be hoped that as our testing techniques are improved and our understanding of the nature of fungicidal actions develops, our efficiency in finding new fungicides will increase.

DERIVATIVES OF DITHIOCARBAMIC ACID AS FUNGICIDES[1]

W. H. Tisdale and A. L. Flenner[2]

The ravages of fungi and other pests have been recognized, and the use of chemicals as pesticides has been known at least three thousand years. Progress, however, in the development of fungicides was slow and relatively insignificant until the past few decades. As our supply of virgin land was gradually exhausted and agriculture became more intensified (and transportation facilities expanded) conditions for the spread of fungi were increasingly more favorable, and their ravages increased. We now measure our annual losses in this country due to fungi in the billion dollar category in spite of the control measures used. In recent years as a result of the impact of this situation on modern scientific thought, special attention has been given through well organized research programs, to the chemical as well as the biological and other phases of fungus control. The earlier research was directed chiefly toward the exploratory phases of the causes of the diseases, of molds and decay, and of the effects of the parasite on the host. The development of research techniques was given special attention. Such control measures as were suggested were often very inadequate.

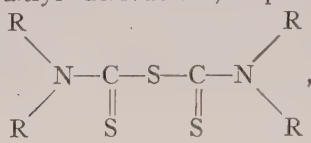
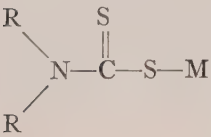
Through the cooperative efforts of the state and federal experimental stations, other public and endowed institutions, and the chemical industry, much has been learned in recent years about the important needs for and usefulness of fungicides. Many of the weak and strong points of the commonly used older fungicides such as copper, sulfur, mercury, formaldehyde, and phenolic compounds have been learned. In addition, new and more effective products have been developed to meet some of the important specific needs. The aim has been not to find just another fungicide but to find better fungicides to replace unsatisfactory products, or products that fill previously unfilled needs. More effective formulations of the old established fungicides often supply the answer. Hopes of finding an all-purpose fungicide seem to have given way, to a considerable extent, to the necessity of developing suitable products for specific needs.

In search for better pesticides, Tisdale and Williams (21) in 1931 discovered the fungicidal properties of some of the derivatives of dithiocarbamic acid,  Through the research in the rubber proc-

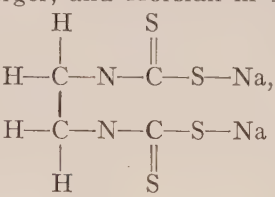
[1] Invitational paper presented before the American Chemical Society in Philadelphia, Pa., on April 12, 1950.

[2] E. I. du Pont de Nemours and Company, Grasselli Chemicals Department, Wilmington, Dela.

essing field where members of this class are used as vulcanization accelerators, many of them were available for evaluation as fungicides and other pesticides. Other derivatives were synthesized for trial. In addition to their fungicidal action, some of these compounds were found to be effective as bactericides, contact insecticides, insect repellents, and miticides.

The alkyl derivatives, especially the methyl and ethyl thiuram sulfides,  , and the metal dithiocarbamates,  , were outstanding in fungicidal effectiveness.

A summary of the early investigations by the du Pont Company and by the Universities of Delaware (9) and Wisconsin (12), and by Harrington of the U. S. Department of Agriculture (11), was given by Tisdale and Flenner (21) in 1942.

This group of dithiocarbamic acid derivatives has now been investigated in several fields of pest control. The extensive work in the fungicidal field has resulted in the established usage of a number of thiuramsulfide and metal dithiocarbamate derivatives as fungicides. A new series of effective derivatives, the ethylene bisdithiocarbamates, was uncovered by Dimond, Heuberger, and Horsfall in 1941 (6). Disodium ethylene bisdithiocarbamate,  , and the corresponding zinc salt have high fungicidal efficiency.

The extensive research on the dithiocarbamic acid compounds has resulted in the development of what is now considered by many plant pathologists and growers to be the most versatile and effective group of organic fungicides. This discovery in the fungicidal field may be compared with the discovery of DDT in the insecticidal field. It preceded DDT by several years but came in the midst of a severe depression when the interest in fungicides and other such products was at a low ebb, while DDT appeared at a most opportune time. While the du Pont Company was pursuing the laboratory and field work on these products as pesticides, many other investigators were also working with them. In England, Moore, Montgomery and Shaw (18), Montgomery and Moore (17), and Marsh (15) investigated the thiuramsulfides as fungicides and found the disul-

fides to be more effective than the monosulfides in the laboratory and field.

One of the most extensive of the earlier investigations was that of Goldsworthy, Green and Smith (7) of the U. S. Department of Agriculture. Results of experiments to determine the fungicidal and phytocidal properties of some metal dialkyldithiocarbamates, including the sodium, ferric, lead, zinc, copper, silver, and mercury dimethyl, diethyl, and dibutyldithiocarbamates, were reported by them. According to this report, the dimethyl derivatives appeared to possess the greatest fungicidal value while the dibutyl derivatives possessed the least. The iron, lead, and zinc dimethyl derivatives appeared to offer the greatest promise, since these caused the least plant injury. Hamilton and Palmiter (10) were among the first investigators to report the successful use of ferric dimethyldithiocarbamate as a plant spray fungicide. They found this product to be highly effective for the control of apple rust (*Gymnosporangium juniperae-virginianae* Schw.). It also showed promise for the control of apple scab (*Venturia inaequalis* [Cke.] Wint.) and cherry leaf spot (*Coccomyces hiemalis* Higgins). In 1943 Dimond, Heuberger and Horsfall (6) reported on the fungicidal value of the ethylene bisdithiocarbamates which they found to be more effective against some species of fungi than the dimethyl series.

Literature on this subject is extensive (1, 2, 5, 14). Only those reports pertinent to this discussion are listed.

Common names have been assigned to the active compounds contained in the commercial products developed from this class of compounds by the American Phytopathological Society in cooperation with the U.S.D.A. Interdepartmental Committee on Pest Control (20). These are: *thiram* (tetramethylthiuramdisulfide); *ferbam* (ferric dimethyldithiocarbamate); *ziram* (zinc dimethyldithiocarbamate); *nabam* (disodium ethylene bisdithiocarbamate); *zineb* (zinc ethylene bisdithiocarbamate).

Some of these products have promising potential uses outside of the agricultural field. Ziram is being used for the treatment of fabrics and other commercial items for mold control. Tetramethylthiuram monosulfide and the corresponding ethyl derivative have proved effective for the control of certain fungus and mite infections of the skin of humans and domestic animals (8, 13). However, as is true with sulfur and some of its compounds, tetramethylthiuram monosulfide, tetramethylthiuramdisulfide, and some other members of this class cause dermatitis and irritation of the nose and throat of some humans. Tetraethylthiuram monosulfide, which, in investigations in the United States and England, caused practically no irritation of any kind (8, 13), is in limited commercial use for the control of scabies and fungus infections of the skin. Some of the dithiocarbamates are also known to cause irritation of the skin and mucous membranes of nose and throat. With reasonable care such irritation can be avoided.

The specific action of these compounds as fungicides makes it necessary to manufacture a number of products to meet the important needs where

a single product would be much preferred if a suitable one could be found. However, the advantages of these specifics in the way of more effective disease control and of their safety to plants, and of the resulting higher yields of disease-free crops, amply justify their production and use.

Some of these compounds repel insects, especially beetles such as the Mexican bean beetle, Japanese beetle, and grain weevils. Thiram, ferbam, and ziram repel animals such as rats, rabbits, and deer when applied to their food plants or products. These are sometimes valuable adjuncts to their functions as fungicides.

THIURAM SULFIDES

Thiram has found its most effective use as a seed and soil disinfectant. It prevents seed decay and damping off of seedlings and is safe to the seed of most varieties even when used in overdoses. It has practically replaced the more hazardous organic mercury compounds as the leading corn seed treatment.

Thiram has also fulfilled a long-felt need for a good peanut seed protectant. It now appears to offer considerable promise as a soil treatment, applied at the rate of 3 to 4 lb. per acre, for the control of root rot (*Aphanomyces* spp.) of sugar beets, a very important disease in the West. When applied to onion seeds as a coating with an adhesive it protects the plants against soil-inhabiting smut (*Urocystis cepulae* Frost) infections. It also has promise for the treatment of certain vegetable seeds, rice, and sorghums. It has an added advantage of protecting seeds against some species of insects in storage.

For the control of brown patch (*Rhizoctonia solani* Kühn) of turf grasses, thiram is the leading treatment. Its fungicidal efficiency combined with a high degree of safety to the grasses makes it especially attractive for this use. For the control of dollar spot and snow mold of turfs, other products including some of the mercurials and a cadmium containing inorganic complex are more effective.

DITHIOCARBAMATES

Members of the dimethyl and ethylene bis series have proved to be outstanding spray and dust treatments. Ferbam is especially useful in fruit disease control (10, 21). It has no equal for the control of apple rust (*Gymnosporangium juniperae-virginianae*) and quince rust (*Gymnosporangium clavipes* C. & P.). Under many conditions it is a superior product for the control of apple scab, especially on the fruit. It is somewhat less effective on the foliage. In some severely infested areas it may be used only in a part of the spray schedule, or in combination with sulfur for best results for scab control. Pear scab (*Venturia pyrina* Aderhold) is very effectively controlled with ferbam, and it does not cause the russeting of fruit often caused by other fungicides in the Northwest.

Ferbam has an advantage in that it can be used with oil-lead arsenate and oil-nicotine combinations where sulfur cannot be used. It does not cause russeting of fruit of sensitive varieties.

Ferbam is considered a superior product for the control of brown rot (*Sclerotinia fructicola* [Wint.] Rehm.) and leaf spot of sweet cherries, and it is safer to both foliage and fruit than the copper fungicides. It is effective for the control of brown rot of peaches, but is not considered superior to sulfur under most conditions of use for this purpose.

In the eastern states where black rot (*Guignardia bidwellii* [Ellis] V. & R.) and downy mildew (*Plasmopora viticola* [B. & C.] Berl & Dot.) of grapes are very important diseases, ferbam has proved effective for the control of black rot, and for downy mildew, except in severe cases where copper fungicides have been more reliable. Ferbam does not cause stunting of the grapevines, which copper may cause under adverse climatic conditions. Ferbam is the preferred treatment for several important diseases of small fruits. It has provided excellent control of the diseases of some of the bush fruits, especially for the fruit rots of cranberry. In some localities ferbam is still preferred for the control of certain vegetable crop diseases such as celery blights and downy mildew (*Peronospora spinaciae* Laub.) of spinach, although zineb is superior for the control of these diseases under most conditions. Tobacco blue mold (*Peronospora hyoscyami* de By.) and black spot (*Diplocarpon rosae* [Fr.] Wolf F. A.) of roses are two other important diseases that are effectively controlled with ferbam.

Ziram has found its greatest uses in the field of vegetable crop disease control. Outstanding uses are the control of tomato anthracnose and the early blight of tomato and potato. It has been ineffective against late blight of these two crops. It is effective for the control of such cucurbit diseases as anthracnose (*Colletotrichum lagenarium* [Pass.] Ell. & Hals.) and downy mildew (*Pseudoperonospora cubensis* [B. & C.] Rostowzew). It is superior to the copper fungicides for the control of anthracnose and equal to them for downy mildew of cucurbits. Less plant injury is caused, and better yields are obtained with ziram than with the copper fungicides. It also is effective for the control of downy mildew of spinach, anthracnose (*Colletotrichum lindemuthianum* [Sacc. & Magn.] Bri. & Cav.) and rust of snap beans and lima beans.

Ziram is about the equal of sulfur for controlling brown rot of peaches. It also offers promise for the control of scab and leaf curl (*Taphrina deformans* [Berk.] Tul.) of peaches. Ziram can be used safely on plants except the zinc-sensitive species. It has proved nutritionally beneficial to plants grown in zinc-deficient soils. A special formulation of ziram is useful for the control of mildew of fabrics and other cellulosic materials under warm, humid conditions.

Zineb and nabam are members of the ethylene bis series of dithiocarbamates. Zineb is the zinc salt and nabam the corresponding sodium salt.

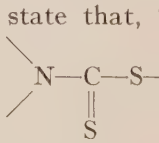
The latter is generally mixed in the spray tank with zinc sulfate or zinc sulfate and lime. If zinc sulfate alone is used, reaction takes place readily in the tank to precipitate zinc ethylene bisdithiocarbamate. The addition of lime to the zinc sulfate-nabam system results in the precipitation of zinc hydroxide leaving the dithiocarbamic acid salt in solution. When a thin film of this mixture is sprayed on the foliage, zineb is formed by the action of CO_2 from the air. Any unreacted sodium salt would tend to have a more rapid fungicidal action if present. The sodium salt, however, is less stable, highly water soluble, and more phytocidal than the zinc salt, and, consequently, less desirable for use on plants. It is now believed that there is no advantage in using lime in the mixture. Sufficient zinc sulfate is added to complete the reaction, and the end result is essentially the same as if zineb were applied.

The outstanding use of zineb and the nabam-zinc sulfate combination is for the control of late blight (*Phytophthora infestans* [Mont.] de By.) of potatoes and tomatoes. It also controls early blight (*Alternaria solani* [Ell. & G. Martin] L. R. Jones & Grout) and other leaf infections of these crops. Where both early and late blights occur in epidemic form, zineb is the outstanding treatment. The early and late blights of celery also are controlled with these fungicides. Other diseases effectively controlled with zineb and nabam are downy mildew and white rust of spinach, snapdragon rust, petal blight of azalea and camellia, anthracnose and rust of beans, onion purple blotch, and certain diseases of gladiolus.

Although ethylene bis compounds are replacing other fungicides to a considerable extent for the control of vegetable crop diseases, they are relatively new in the field of fungicides, and it remains to be seen as to how extensive they may be used eventually.

MECHANISM OF FUNGICIDAL ACTION

If the mechanism of fungicidal actions of chemicals were known, we would, no doubt, be in a better position to select effective classes of compounds for trial. So far, work on this subject is principally in the theory stage. Theories are valuable in that they present definite challenges to the investigator. Several theories have been advanced to explain the fungicidal action of the derivatives of dithiocarbamic acid. None of these, however, adequately explains the wide range of fungicidal effectiveness or specific differences in activity of different members of this class of compounds.

Goldsworthy, Green and Smith (7) state that, "... the fungicidal action does not clearly indicate that the  group *per se* is responsible for the fungicidal properties, since all compounds possessing this group are not fungicidal." They also state that there is some indication that, in the case of derivatives of dithiocarbamic acid, fungicidal activity

may be correlated with water solubility but that a few contradictory results more or less invalidate this theory, pointing out that some entirely water-soluble compounds differ in their activity. Barrett and Horsfall (3) postulate that the dithiocarbamates may interrupt some biological activity of the fungus by: (a) spontaneously liberating H_2S which reacts with the protein of the spores, and (b) inactivating the trace elements which the fungus needs in its metabolism. Parker-Rhodes (19) says, ". . . it is inferred that: (a) dithiocarbamates are decomposed by the spores to a greater or lesser degree into amines and carbon disulfide, both being toxic; (b) the amines can be absorbed only in a combined form which may be the dithiocarbamate itself but is more probably a derivative, possibly an ester; (c) thiuram sulfides act through being decomposed to dithiocarbamates."

Miller and Elson (16) state, "In connection with the relation of structure to activity the data obtained indicate that with each type of derivative—dithiocarbamate, thiuram monosulfide, and thiuram disulfide—the lower members, i.e., the methyl and ethyl compounds, are the most active and the higher alkyl derivatives are relatively inactive. Thus, as a general rule, in all the three series activity against both bacteria and fungi decreases with the increasing size of the N-substituted groups. This finding is in agreement with the observations of Davies and Sexton (4) in their study of the activity of a number of these compounds against plant pathogens." Miller and Elson mention water solubility as a possible determining factor. With regard to mechanism, they state, "Although little can be concluded regarding the role of specific parts of the molecule in determining activity beyond that given above, it is interesting to note that all the compounds showing significant antibacterial activity contain the —C(=S)—S— group, but the miscellaneous derivatives of thiourea studied which contain the —C(=S)— group are relatively inactive."

The difference in effectiveness of the active compounds on different organisms must be considered. This is especially true in the case of fungi. Miller and Elson state, "Thus, whereas both tetramethyl mono- and disulfide were comparable in activity against the dermatophytes *Trichophyton gypseum* and *Epidermophyton floccosum;* against *Sporotrichum schenckii* and *Candida albicans* the disulfide was highly active, but the monosulfide was relatively low in activity. Such differences in activity in these closely related compounds would indicate that they exert their inhibitory effects, at least against some organisms, by different mechanisms."

There is not yet sufficient evidence to justify firm conclusions concerning the mechanism of fungicidal and bactericidal action of the derivatives of dithiocarbamic acid. A review of the results obtained with a large number of compounds shows that certain factors are essential for fungicidal

activity. All the known active members of the class possess some degree of water solubility, although this is very slight in case of some of the most active compounds such as tetramethylthiuramdisulfide and ferric dimethyldithiocarbamate.

Some of the more highly water-soluble compounds are less effective than these. All entirely water-soluble products are not equally effective. Active, highly water-soluble products, such as sodium dimethyldithiocarbamate, may act more readily as a result of the solubility. We have no conclusive proof that water solubility is necessary for fungicidal action. Such relatively insoluble compounds as those mentioned above and other chemicals, e.g., mercurous chloride and copper carbonate which are highly insoluble, are valuable fungicides. It is theorized that these compounds may be brought into solution by the excretions of the fungi.

Other factors such as structure and stability, no doubt, play a part in fungicidal action. In water solution or possibly in solution in the excretions of the fungi, the compounds may become ionized to form the dithiocarbamate ion $=N-\overset{\overset{\|}{S}}{C}-S-$. This ion may penetrate the cell wall of the spore and then interrupt the biological activity of the fungus by liberating CS_2, H_2S, or by other means. The compounds as such may penetrate the fungus and react with the cell contents, or, it may decompose after penetration, and the decomposition products such as H_2S may function as the active agents. Structural differences may influence such factors as solubility and stability and thereby influence penetration, decomposition and degree of activity. The methyl derivatives are more effective than the ethyl which in turn are more effective than the propyl or butyl derivatives. These differences in activity are possibly due to the fact that the lower members of the series are the least stable. They may penetrate the tissues of the fungus more readily and react with the cell constituents, or they may decompose more readily to release by-products which are active.

Different species or even different strains of fungi may respond differently to the same compound regardless of the degree of its solubility or the degree of stability. The constituents of the fungus may be such as to influence penetration or reactions with the chemical to produce lethal action once it has penetrated.

It is difficult to visualize any simple theory that may prove valid in explaining the mechanisms of the fungicidal actions of these compounds. The available evidence indicates that the fungicidal action of the dithiocarbamic acid derivatives is inherent in the dithiocarbamate ion, $=N-\overset{\overset{\|}{S}}{C}-S-$. The mechanism or mechanisms of action of products containing this grouping have not as yet been clearly defined.

LITERATURE CITED

1. AMERICAN PHYTOPATHOLOGICAL SOCIETY. VEGETABLE SEED TREATMENT SUBCOMMITTEE OF THE SEED TREATMENT COMMITTEE. Studies on vegetable seed treatments in 1943; 1944. Plant Disease Reptr. Suppl. **145**. 97 pp. 1944; Suppl. **161**. 66 pp. 1946.
2. AMERICAN PHYTOPATHOLOGICAL SOCIETY. FUNGICIDE COMMITTEE, SUB-COMMITTEE ON "SUMMATION OF THE PERFORMANCE OF NEWER FUNGICIDES." Nation-wide results with fungicides in 1948—Fourth Annual Rept. Plant Disease Reptr. Suppl. **181**. 87 pp. 1949.
3. BARRETT, RAYMOND W., and JAMES G. HORSFALL. Fungicidal action of metallic alkyl bisdithiocarbamates. Connecticut Agric. Exp. Sta. Bull. 508. 51 pp. 1947.
4. DAVIES, W. H., and W. A. SEXTON. Chemical constitution and fungistatic action of organic sulfur compounds. Biochem Jour. **40**: 331–334. 1946.
5. DIETZ, H. F. List of publications on "fermate" fungicide from Sept. 11, 1934 to July 1, 1944. Mimeographed, 9 pp. E. I. du Pont de Nemours & Company, Inc., Grasselli Chemicals Department, Wilmington 98, Delaware. 1944.
6. DIMOND, ALBERT E., JOHN W. HEUBERGER, and JAMES G. HORSFALL. A water soluble protectant fungicide with tenacity. Phytopath. **33**: 1095–1097. 1943.
7. GOLDSWORTHY, M. C., E. L. GREEN, and M. A. SMITH. Fungicidal and phytocidal properties of some metal dialkyl dithiocarbamates. Jour. Agric. Res. **66**: 277–291. 1943.
8. GORDON, R. M. Observations on the treatment of scabies. British Med. Jour. **1942** (4248): 685–687. June 6, 1942.
9. GUY, H. G. Investigation of organic compounds as insecticides. Delaware Agric. Exp. Sta. Bull. 206. 60 pp. 1937.
10. HAMILTON, J. M., and D. H. PALMITER. Fermate—a promising new fungicide. Farm Research **9** (2): 14. 1943.
11. HARRINGTON, GEORGE E. Thiuram sulfide for turf diseases. Science **93**: 311. 1941.
12. HERRICK, C. A., C. E. HOLMES, and D. L. DEGIUSTI. The experimental use of organic sulfur compounds for the prevention of cecal coccidiosis in chickens. Jour. Vet. Res. **3**(6): 117–127. Jan. 1942.
13. JENNINGS, S. The treatment of mange by tetraethylthiuram monosulfide. Vet. Record-London-W.C.I. No. 2823. Aug. 15, 1942.
14. MCCALLAN, S. E. A. Dithiocarbamate fungicides. Agric. Chem. 1(7): 15–18, 55. 1946.
15. MARSH, R. W. Some applications of laboratory biological tests to the evaluation of fungicides. Ann. App. Biol. **25**: 583–604. 1938.
16. MILLER, CATHERINE R., and WILLIAM O. ELSON. Dithiocarbamic acid derivatives. I. The relation of chemical structure to *in vitro* antibacterial and antifungal activity against human pathogens. Jour. Bact. **57**: 47–54. 1949.
17. MONTGOMERY, H. B. S., and M. H. MOORE. A laboratory method for testing the toxicity of protective fungicides. Jour. Pomol. & Hort. Sci. **15**: 253–266. 1938.
18. MOORE, M. H., H. B. S. MONTGOMERY, and H. SHAW. Field trials in 1936 of the fungicidal and phytocidal properties of certain new chemical preparations. A progress report. I. MOORE, M. H., and H. B. S. MONTGOMERY. Fungicidal properties. II. SHAW, H. Preliminary phytocide tests. East Malling (Kent) Res. Sta. Ann. Rept. **24**: 259–266. 1936.
19. PARKER-RHODES, A. F. Studies in the mechanism of fungicidal action. V. Non-metallic and sodium dithiocarbamic acid derivatives. Ann. App. Biol. **30**: 170–179. 1943.
20. ROHWER, S. A. Notice. Phytopath. **40**: 118. 1950.
21. TISDALE, W. H., and A. L. FLENNER. Derivatives of dithiocarbamic acid as pesticides. Indus. & Eng. Chem. **34**: 501–502. 1942.

FUNGITOXICITY OF HETEROCYCLIC NITROGEN COMPOUNDS[1]

James G. Horsfall and Saul Rich[2,3]

Beginning with certain pyrrole derivatives in early 1938, we have been interested sporadically in the fungitoxicity of heterocyclic nitrogen compounds. During the intervening time, some 165 compounds have been investigated, some thoroughly, some partially. The data have been gathered for this paper.

Only a few compounds have been synthesized especially for the study. In general, we have depended upon compounds purchased from Eastman Kodak Company, or obtained from various industrial research laboratories.

The compounds have reasonable purity, but since melting point determinations were not made, we cannot vouch for purity.

OBJECTIVES

Data will be presented on the relation of structure and activity of heterocyclic nitrogen compounds to fungitoxicity. The data so far obtained suggest that most unsubstituted heterocyclic nitrogen compounds are not active on spores. Certain types of substituents, however, make them active. Pyridine, for example, has no significant effect on spores. If, however, a styryl group is attached in the 2-position (2-stilbazole), the compound becomes very active. From various experiments, we submit that this is related to availability (10) factors. Pyridine presumably does not penetrate the spore. If, however, a lipophilic group is added to the pyridine molecule, it can permeate the spore and kill it.

Of course, the polar groups such as —OH,—SH,—COOH, and others add fungitoxicity to heterocycles as they do to other nuclei.

TECHNIQUE

In all cases we have used the spore germination method of assaying potency of the test compounds (10). The other common method of assaying chemicals by mixing the compound with the food on which the fungus is to be grown, was not used because the test chemical may react with the food as well as with the fungus. In the spore germination test, one has a reasonably pure system of fungus and chemical.

Two test fungi were used: *Sclerotinia fructicola* (Wint.) Rehm and *Ma-*

[1] Invitational paper presented before the American Chemical Society in Philadelphia, Pa., on April 12, 1950.

[2] The Connecticut Agricultural Experiment Station, New Haven 4, Connecticut.

[3] The authors extend their thanks to Mrs. Elinor Moquet for excellent technical assistance.

crosporium (lately called *Stemphylium*) *sarcinaeforme* Cav. The former produces the brown rot disease of ripe peaches and plums; the latter produces an inconspicuous leaf spot on red clover. A brief description of the assay technique follows. All compounds are dissolved in a suitable solvent and 0.2 ml. is pipetted with a graduated syringe to the cylindrical depression in a so-called culture slide made of glass. This depression is 14 millimeters in diameter and 2.5 millimeters deep. It holds 0.4 milliliter. The solvent is allowed to evaporate. Then the depression is filled level full with 0.4 ml. of standard spore suspension containing 50,000 spores per ml. of redistilled water. If the depression is level full, the spore drop presents no optical difficulties when the spores are counted.

The spores are incubated overnight at 25° C., and examined next morning with a binocular microscope. The percentage of spores not germinating is recorded. The one or two per cent of natural mortality is usually disregarded.

In the initial screening of compounds, the dosages are in steps of 10-fold. The compound is dissolved in the solvent at 10, 1, 0.1 and 0.01 grams per liter. When placed in the depression, this gives 1300, 130, 13, and 1.3 μg. per cm.^{2} of surface after drying. Since the dose ratio is 10, this test gives only a rough distinction between the sheep and the goats among the compounds. We will, however, make much use of the data from the test. In general we shall refer to the toxicity as plus or minus. Plus means that more than 50 per cent of the spores failed to germinate at 1 gram per liter or 130 μg./cm.^{2}; minus, of course, means that fewer than 50 per cent failed to germinate. The symbol $\pm$ means that practically all spores failed to germinate at 10 g./l. or 1300 μg./cm.^{2} and practically none failed at 1 g./l. or 130 μg./cm^2. *M* and *S* will be used to indicate the test fungi, *M. sarcinaeforme* and *S. fructicola*, respectively.

Interesting compounds receive further tests. The dose ratio for the later tests is 2 or even $\sqrt{2}$. This spaces the doses close enough together to give more precise data. Treatment is otherwise the same. Sometimes this second test is made by spraying suspensions or solutions from an isopropyl alcohol/water mixture onto glass microscope slides coated with cellulose nitrate (10). From data thus obtained, we can plot dosage-response curves (10).

If the data on dosage and response are plotted on a logarithmic-probability grid, a straight line usually results. Such a line has two qualities that are useful in bioassay of chemicals—position and slope (10). Position is usually recorded as ED50, that is, the effective dosage in micrograms (μg.) per square centimeter of surface that is required to inhibit 50 per cent of the spores. Some workers record position as the dose for least response or dose for maximum response. This is an older but much less precise method of expression. The ED50 term can be determined more accurately as witness any modern discussion of the mathematics of bioassay.

ED50 is a measure of potency—How good is the compound? This is

the classical measure of toxicity, but it is inadequate, because it gives no hint of mechanism. Slope gives an indication of mechanism of action. Slope indicates whether two compounds are killing the organism by the same or by different mechanisms. Suppose a new toxicant is synthesized. If its dosage-response curve shows the same slope as another analog, it is probably acting by the same mechanism. If the slope is different, one can assume that a different mechanism is being affected.

Slope is calculated as the change in probit mortality with unit change in log dose. Hence, the larger the number, the steeper the slope. Slope may be expressed by the well-known function of $\Delta y/\Delta x$. Slope can be derived easily from the dosage-response curve as plotted on log-probit paper. The formula as suggested by Gaddum (8) is:

$$\text{slope} = \frac{1}{\log \text{ED84} - \log \text{ED50}}.$$

In presenting a paper of this type, one is always confronted with the question of whether the treated spores are dead or merely inhibited. To some extent this is academic because the answer depends upon what is used to resuscitate the spores. Leaching 8-quinolinol-treated spores with water will cause them to revive, for example. A metal-treated spore, leached with a sulfhydryl compound, will recover. In practice, recovery cannot occur as long as the spore remains in contact with the toxicant. To avoid conflict of terminology, we shall use fungitoxic rather than fungicidal or fungistatic. Oster and Golden (22), recognizing the problem, use antifungal.

EXPERIMENTAL

The numerical data for all compounds are given in Tables I and II. The compounds are arranged alphabetically. The results will be discussed in terms of the activity of the unsubstituted heterocyclic compounds and of the effect of various substituents on their activity. This method of presentation seems preferable to that of discussing each heterocycle in turn, together with its substituents.

An effort has been made to present structural formulas for all critical compounds. In many cases the whole formula is given or reference is made to the nucleus from which it could be easily constructed by anyone interested.

FUNGITOXICITY OF UNSUBSTITUTED HETEROCYCLES

Perhaps the most striking feature of the data is that so few of the unsubstituted heterocyclic nitrogen compounds display activity. It is well to note that we do not say that they are not fungitoxic, merely that they do not display activity. As will turn out below, this is more than semantics. Groups, such as hydrocarbons, that are not toxic in themselves may impart activity to a heterocycle.

TABLE I

EFFECT OF HETEROCYCLIC NITROGEN COMPOUNDS ON SPORE INHIBITION

Compound	Per cent inhibition at various dosages in μg./cm.²											
	Macrosporium sarcinaeforme						*Sclerotinia fructicola*					
	Depression slides				Sprayed slides		Depression slides				Sprayed slides	
	1300	130	13	1.3	ED50	Slope	1300	130	13	1.3	ED50	Slope
1-Acetyl-2-thiohydantoin	100	66	22	3			100	98	2	0		
Acridan	100	46	2	0	>50		100	0	3	0	>50	
Acridine	100	100	97	5	9.6	4.2	100	100	100	38	3.3	4.6
Acridone	100	100	35	0	28	8.8	100	100	51	1	33	7.4
Actidione	100	100	100	50	1.0	9.4	100	100	100	100		
2-Amino-4-(*p*-diphenylyl) thiazole	0	5	0	0			0	5	0	0		
1-(2-Aminoethyl)-2-heptadecyl-2-imidazoline					0.3	6.6						
2-Aminonaphtho[2.1]thiazole	50	50	0	0			100	0	0	0		
4-Amino-2-phenyl-1,5-dimethyl-pyrazolone	15	4	2	0			21	3	2	0		
5-Amino-2-phenyl-2-*v*-triazolo[d]-pyrimidin-7-ol	11	16	16	6			0	0	0	0		
3-Aminophthalhydrazide	0	0	0	0			0	0	0	0		
2-Aminothiazole	100	50	0	0			100	0	0	0		
6-Aminouracil	7	0	1	0			0	0	0	0		
2-Amyl-2-imidazoline	100	50	0	0	15.2	9.4	100	50	0	0		
1-(*p-tert*-Amylphenyl)-3,5-dimethyl-4-nitrosopyrazole					0.4	10.0					0.2	7.0
2-(*n*-Amyl)pyridine	0	0	0	0			0	0	0	0		
4-(*n*-Amyl)pyridine	20	0	0	0			100	0	0	0		
N-[β-(4-Antipyrylamino)-crotonyl]aniline	0	0	0	0			0	0	0	0		
o-Benzoic sulfimide	100	100	100	5	2.8	5.7	100	37	6	0		
o-Benzoic sulfimide (sodium salt)	0	0	0	0			0	0	0	0		
10-Benzoyl-3,7-bis(dimethyl-amino)phenothiazine	—	8	5	3			—	—	6	5		
1-(2-Butylaminoethyl)-2-hendecyl-2-imidazoline	100	100	100	5			100	100	100	100		
Carbazole	0	0	0	0			0	0	0	0		
2-Chloro-x-nitrobenzothiazole					10.8	2.2						
8-Chloro-5-nitroquinoline	100	100	50	0			100	100	50	0		
7-Chloro-2,2,4-trimethyl-1,2-dihydroquinoline	2	5	4	0			100	100	41	6	24.5	0.9
1-(2-Cyanoethyl)-L-pyroglutamic acid	100	100	100	6			100	100	5	0		
cis-Decahydroquinoline	100	85	0	0			100	100	0	0		
2,6-Diamino-5-nitroso-4-pyrimidol	—	23	32	17			—	0	1	0		
2,6-Diamino-4-pyrimidol sulfate	100	100	63	11			—	23	51	6		
5,6-Dihydro-6-imino-5-isonitroso-uracil	25	5	3	1			0	0	0	0		
1,4-Dihydro-1-methyl-4-quinolinethione	?*	9	2	3			85	31	16	3		
9,9-Dimethylacridan	2	3	7	2	>50		100	100	99	4	3.1	6.6
5-(*p*-Dimethylaminobenzylidene)-rhodanine		100						100			16.3	3.2
3,5-Dimethyl-4-nitrosopyrazole					16.9	15.2					9.6	7.4
2,5-Dimethylpyrrole	100	50										
4,5-Dimethyl-2-thiazolethiol	100	50	0	0	>25		100	100	0	0	>25	
α,α′-Dipyridyl	100	7	0	0			100	98	0	0		
2,6-Distyrylpyridine	35	0	0	0			0	0	0	0		
7-Ethoxy-3-hydroxyphenothiazine	0	0	0	0			100	33	17	5		
3-(*p*-Ethoxyphenylamino)-phenothiazine	100	100	7	2			100	100	30	4		
2-Hendecyl-2-oxazoline	100	100	100	46			100	100	31	0		
2-(12-Heneicosenyl)-1-(2-hydroxy-ethyl)-2-imidazoline	100	100	100	0			100	100	100	56		
2-(8-Heptadecenyl)-1-(2-hydroxy-ethyl)-(4 or 5)-methyl-2-imidazoline	100	100	100	56			100	100	100	100		
2-Heptadecyl-2-imidazoline					0.3	3.7						
1-(2-Hydroxyethyl)-2-heptadecyl-2-imidazoline					1.2	1.4						
1-(2-Hydroxyethyl)-2-pentacosanyl-2-imidazoline	100	50	0	0			100	0	0	0		
1-(2-Hydroxyethyl)-2-tridecyl-2-imidazoline					0.1	10						
3-Hydroxyphenothiazine	54	15	3	1			9	1	1	0		
2-Imidazolidinethione	25	4	2	0			39	5	4	3		
Indole	100	58	47	43			100	87	3	1		
7-Iodo-8-quinolinol-5-sulfonic acid		100			7.6	7.0		0				
Isoquinoline	100	5	5	1			100	0	0	0		
3,4-Lutidine	0	0	0	0		2.6	0	0	0	0		

TABLE I—*Continued*

Compound	Per cent inhibition at various dosages in μg./cm.²											
	Macrosporium sarcinaeforme						*Sclerotinia fructicola*					
	Depression slides				Sprayed slides		Depression slides				Sprayed slides	
	1300	130	13	1.3	ED50	Slope	1300	130	13	1.3	ED50	Slope
2-Mercaptobenzothiazole		100			8.4	4.6		100				
2-Mercaptobenzothiazole, zinc salt					8.0	4.8						
Methylene blue		100			1.3	9.4		100			>15	
1-Methyl-3-hydroxyphenothiazine	100	11	5	8			100	100	52	2		
2-Methyl-2-imidazoline	100	50	0	0	46.5	8.8	100	50	0	0		
3-Methyl-5-phenyl-4-nitroso-pyrazole					8.1	11.5					0.1	12.5
5-Methyl-2-phenyl-3-pyrazolone	15	7	3	1			100	14	0	0		
2-Methyl-4-quinazolinol	50	43	7	1			5	3	4	3		
8-Methylquinoline	83	5	2	3			100	0	0	0		
Morpholine-4-carboxylic acid, ethyl ester	50	0	0	0			50	0	0	0		
Neutral red, hydrochloride		100			Ca 2.5			100			<1.5	
Nicotinic acid	100	100	7	4			18	16	3	3	>30	
Nicotinic acid, amyl ester	100	63	0	0			100	100	0	0		
Nicotinic acid, butyl ester	100	13	0	0			100	91	0	0		
Nicotinic acid, cyclohexyl ester	100	53	6	4			100	100	6	0	43.8	11.5
Nicotinic acid, decyl ester	4	4	0	0			23	0	2	0	>30.0	
Nicotinic acid, ethyl ester	0	0	0	0			0	0	0	0		
Nicotinic acid, hexyl ester	77	10	9	0			100	62	18	0		
Nicotinic acid, octyl ester	12	7	1	0			100	100	100	0	17.4	6.0
"Orthocide 406" or "SR 406"	100	100	100	48	3.6	7.4	100	100	100	100	2.3	5.2
Phenanthr[4.3]oxazole	0	0	0	0			2	0	5	0		
Phenazine	36	18	8	0			100	98	98	58		
Phenazine oxide	86	23	3	1			100	74	71	37		
Phenothiazine	2	2	0	0			40	12	6	1		
Phenothiazine-5-oxide	0	0	0	0			26	2	0	0		
Phenothiazone					10.4	3.3					0.8	2.5
3-Phenylaminophenothiazine	50	46	30	25			100	60	8	0		
1-Phenyl-3,5-dimethyl-4-amino-pyrazole	100	37	12	4			100	0	0	0		
1-Phenyl-3,5-dimethyl-4-nitroso-pyrazole	100	100	100	100	0.23	11.5	100	100	97	100	1.33	15.2
1-Phenyl-3,5-dimethyl-4-phenyl-azopyrazole	0	3	5	1			0	0	0	0		
2-Phenyl-1,5-dimethylpyrazolone	32	8	2	3			9	1	0	0		
Phthalimide	100	94	7	0			100	27	0	0		
Picrolonic acid	100	100	10	0			100	100	5	1		
Piperidine	0	0	0	0			18	22	10	0		
3-(2-Piperidino-1-hydroxypropyl)-phenanthrene hydrochloride	100	100	75	0			100	100	34	13		
3-(3-Piperidino-1-oxo-propyl)-phenanthrene hydrochloride	100	100	97	24			100	100	85	4		
L-Proline	2	2	0	0			0	0	0	0		
2-Propyl-2-imidazoline	100	0	0	0	Ca 50		100	0	0	0		
Pyridine	1	1	3	1			2	1	0	0		
Pyrrole	100	100	0	0			100	100	0	0		
p-(1-Pyrryl)benzenesulfonamide	6	0	0	0			0	0	0	0		
Quinaldine	100	52	0	0			100	100	17	0		
Quinidine hydrochloride		100			9.1	14.1		0				
Quinine bisulfate		100			5.6	7.4						
Quinine citrate		100			7.3	5.5						
Quinolin-2-amic acid	100	100	100	2			97	22	3	0		
Quinolinamide	0	0	0	0			0	0	0	0		
Quinoline	100	0	0	0			100	0	0	0		
Quinolinic acid	100	59	5	1			100	7	0	0		
Quinolinic acid, dimethyl ester	38	4	0	0			0	0	0	0		
Quinolinimide	100	99	5	0			100	0	0	0		
2-Quinolinol	100	51	37	16			100	2	0	0		
8-Quinolinol		100			Double maximum			100			Double maximum	
8-Quinolinol, calcium salt		100			0.38	3.8		100				
8-Quinolinol, magnesium salt		100			5.3	2.6		100				
8-Quinolinol sulfate		100			12.1	7.4		100				
8-Quinolinol-5-sulfonic acid		100			4.7	5.7		0				
8-Quinolinol-5-sulfonic acid, zinc salt		100			Double maximum			100				
2-Stilbazole	82	94	77	1			100	100	100	0		
Succinimide	100	11	1	54			0	0	0	0		
Sulfapyridine, sodium salt	12	1	0	0			72	0	0	0		
Sulfathiazole	30	24	19	21			10	15	0	7		
3-[3-(1,2,3,4-Tetrahydroisoquinolino)-1-hydroxy-*n*-propyl]-phenanthrene hydrochloride	100	100	100	6			100	100	100	16		

TABLE I—*Continued*

Compound	Per cent inhibition at various dosages in μg./cm.²											
	Macrosporium sarcinaeforme						*Sclerotinia fructicola*					
	Depression slides				Sprayed slides		Depression slides				Sprayed slides	
	1300	130	13	1.3	ED50	Slope	1300	130	13	1.3	ED50	Slope
3-[(1,2,3,4-Tetrahydroisoquinolino)methyl]-4-hydroxy-1,2,3,4-tetrahydrophenanthrene hydrochloride	100	100	28	2			100	100	9	5		
2-[3-(1,2,3,4-Tetrahydroisoquinolino)-1-oxo-propyl]-phenanthrene hydrochloride	100	100	100	25			100	100	90	24		
1,2,3,4-Tetrahydroquinoline	100	0	0	0			100	50	0	0		
2,3,4,5-Tetrakis(4-pyridyl)thiophene	0	0	0	0			50	4	3	4		
Thiobarbituric acid	100	100	100	3	4.8	15.2	100	84	4	2		
Thionine	100	100	100	0	4.2	8.3	100	100	100	7	2.7	8.3
Thionol	?*	13	5	4			?*	1	3	0		
2,5,6-Triamino-4-pyrimidol monosulfate monohydrate	72	4	0	0			0	0	0	0		
N-(Trichloromethylthio)-1,2,3,6-tetrahydrophthalimide ("Orthocide 406")	100	100	100	48	3.6	7.4	100	100	100	100	2.3	5.2
2-Tridecyl-2-imidazoline	100	100	100	50	0.1	9.7	100	100	100	50		
1,3,5-Trimethyl-4-nitrosopyrazole					2.3	10.0					0.1	7.0
2,4,6-Trimethylpyridine	50	0	0	0			0	0	0	0		
2,3,5-Triphenyl-2*H*-tetrazolium chloride	100	100	100	50			100	100	100	0		
N,*N*-1-Tris(2-cyanoethyl)-L-histidine	5	0	0	0			1	1	2	1		
1,2,3-Tris(4-pyridyl)propane	0	0	0	0			100	13	2	5		
2-Undecyl-2-imidazoline	100	100	100	0	0.3	3.4	100	100	100	0		

* The suspension of chemical was so dense that germination was uncertain.

The following heterocycles have consistently shown no inhibition or, at best, weak inhibition of spore germination of *Macrosporium* and *Sclerotinia:* pyridine, piperidine, hexamethylenetetramine, quinoline, isoquinoline, acridan, phenothiazine, carbazole, and benzothiazole. Presumably oxazoline and isoxazole heterocycles also are non-active, since methyl or ethyl derivatives are non-active. Although it is not strictly an unsubstituted heterocycle, phenothiazine-5-oxide may be reported here as non-fungitoxic. The structures of these non-active compounds appear in Figure 1. It is interesting that pyridine acts as a fairly powerful animal poison, still is so inactive on these fungus cells. Thiazole is probably another inactive nucleus. Seven derivatives were tried; only 2-mercaptobenzothiazole, 2-chloro-x-nitrobenzothiazole, and 4,5-dimethyl-2-thiazolethiol were reasonably active. These, of course, bear substituents that are fungitoxic on many nuclei.

Only five unsubstituted nitrogen heterocycles showed activity. These are pyrrole, indole, hydroquinoline (three levels of saturation), phenazine, and acridine. The structures of these compounds appear in Figure 2. Straib (27) reports that acridine is toxic to spores of various rusts.

In comparing the active with the inactive heterocycles, one is struck by the fact that activity is associated with the presence of a hydrogen attached to the nitrogen. Perhaps the most significant comparison is that

between quinoline and the various hydroquinolines. Ordinary quinoline is not active. Hydroquinolines, which have hydrogen on the nitrogen, are active.

It is worthy of note just here that three tetrahydroisoquinolino-phen-

TABLE II

EFFECT OF HETEROCYCLIC NITROGEN COMPOUNDS ON SPORE INHIBITION USING 130 μG. OF RESIDUE PER SQUARE CENTIMETER OF SURFACE AS THE TEST CONCENTRATION

Compound	*Macrosporium sarcinaeforme**	*Sclerotinia fructicola**
Acridine orange	+	+
Acridine yellow, hydrochloride	+	+
2-Aminopyridine	−	−
Benzothiazole	−	−
1,2-Bis(2,5-dimethyl-1-pyrryl)ethane	−	−
2-Chlorobenzothiazole	−	−
8-Chloro-4-chloromethyl-2-quinolinol	−	−
8-Chloro-4-methyl-2-quinolinol	−	−
4-Chloromethyl-2-quinolinol	−	−
Cinchonidine hydrochloride	−	−
Cinchonine hydrochloride	+	+
Cinchonine salicylate	−	+
5,7-Dibromo-8-quinolinol, zinc salt	−	+
2,2′-Dithiobis(benzothiazole)	−	−
Hexamethylenetetramine	−	−
2,6-Lutidine	−	−
2-Methylpyridine	−	−
3-Methylpyridine	−	−
4-Methyl-2-quinolinol	−	−
6-Nitroquinoline	±	±
8-Nitroquinoline	±	+
N-(1-Oxolauryl)phenothiazine	−	−
Pyridoxine hydrochloride	+	−
Quinine bisalicylosalicylate	−	+
Quinine hydrochloride	+	+
Quinine salicylate	+	+
Quinine sulfate	+	+
8-Quinolinol, aluminum salt	+	+
8-Quinolinol benzoate	+	+
Titan yellow	−	−
3,4,5-Triethylisoxazole	−	−
2,2,4-Trimethyl-1,2-dihydroquinoline	−	+
2,4,4-1Trimethyl-2-oxazoline	−	−

* + is total inhibition; ± is partial inhibition; − is no inhibition

anthrene compounds are also quite fungitoxic to both organisms where isoquinoline itself is not active. These three tetrahydroisoquinoline compounds are not listed in Figure 2, because they are not strictly unsubstituted. They, unlike the other hydroquinolines, have no —NH groups, but they do have carbonyl and hydroxyl groups. The phenanthrene moiety probably enhances the toxicity.

Of course, it is true that hydroquinoline has more hydrogen in the ring than quinoline, but we doubt that this accounts for the activity.

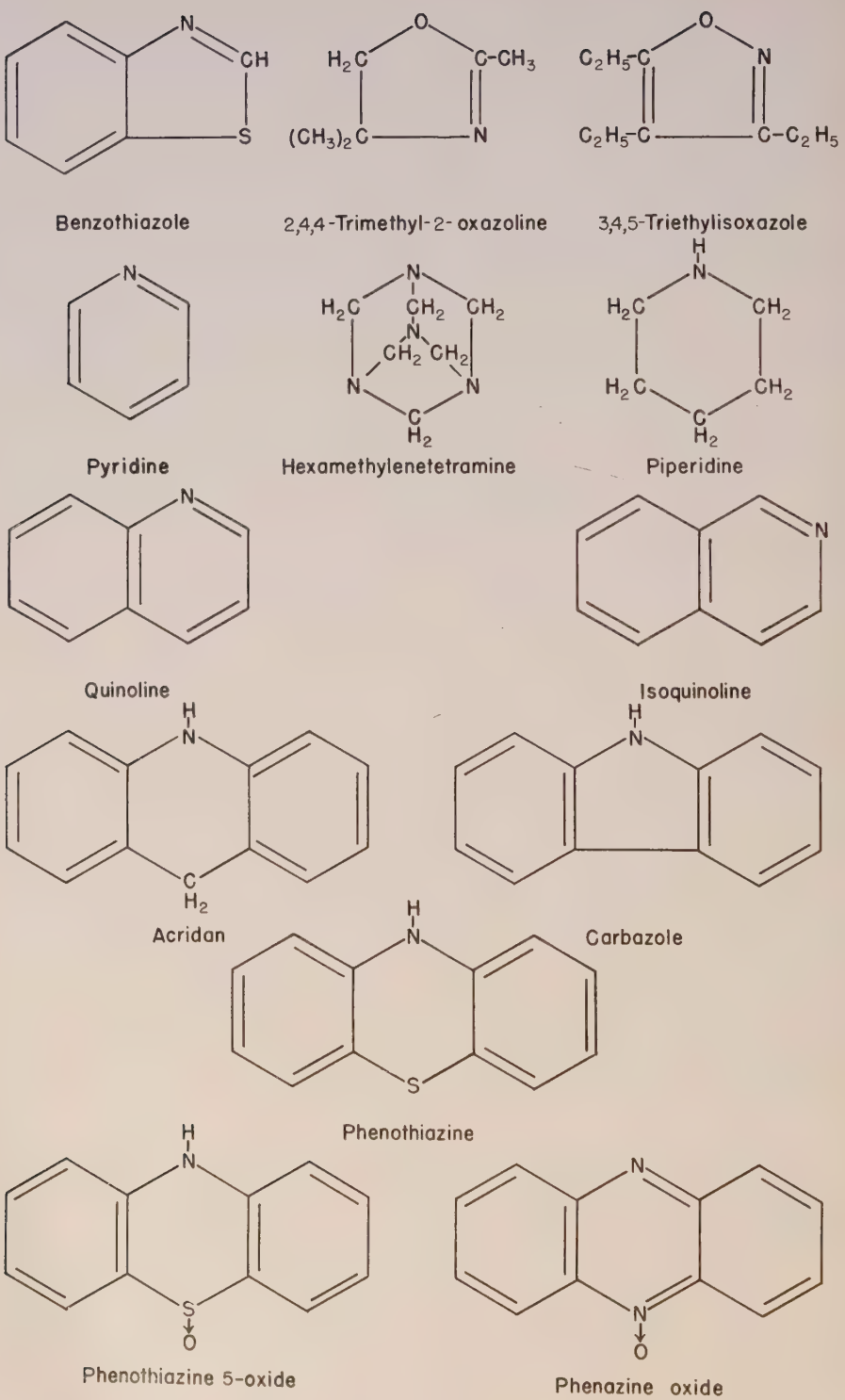


FIGURE 1. Unsubstituted heterocycles that are inactive.

The simplest method perhaps to test the significance of the —NH— group is to substitute it. We have two such compounds, both pyrrole derivatives, 1,2-bis(2,5-dimethyl-1-pyrryl)ethane and *p*-(1-pyrryl)benzenesulfonamide (see Fig. 3). Neither was toxic. Hence, substitution of the hydrogen on the

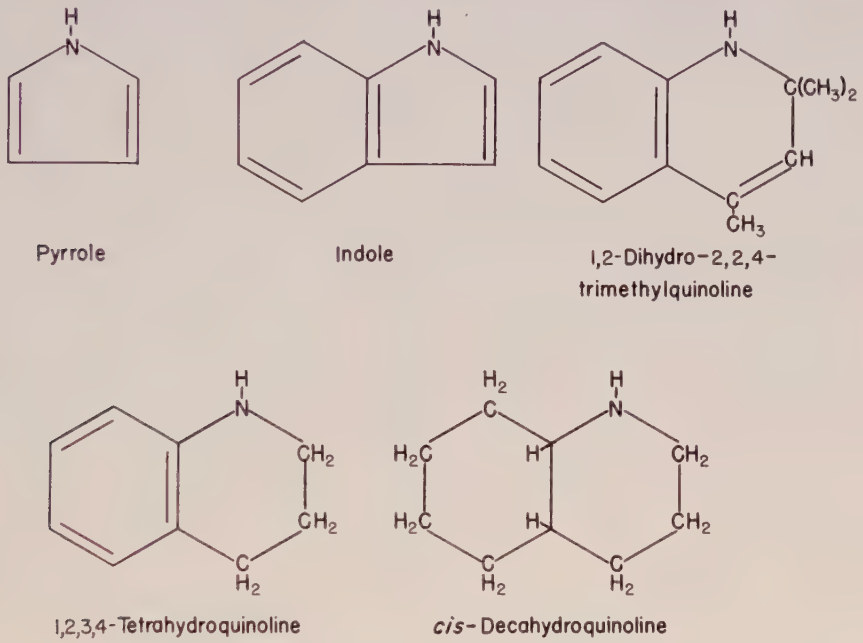


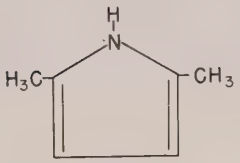
FIGURE 2. Unsubstituted heterocycles that are active.

nitrogen quenches the activity of pyrrole. These data suggest that hydrogen on the nitrogen is a more important toxophore than hydrogen in the ring.

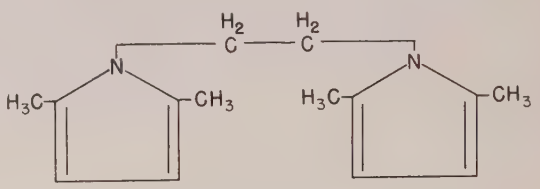
5-Methyl-2-phenyl-3-pyrazolone, *M*−, *S*+, (Fig. 9) offers some confirmation of this. Nitrogen in the 1-position in this compound has a hydrogen attached; it shows some toxicity to *Sclerotinia*. If the nitrogen is substituted with methyl, even this toxicity is quenched (2-phenyl-1,5-dimethyl-pyrazolone, *M*−, *S*−).

There are only two exceptions to the generalization about the hydrogen on the nitrogen: piperidine and the condensed 3-ring compounds. None is toxic despite having a hydrogen attached to the nitrogen.

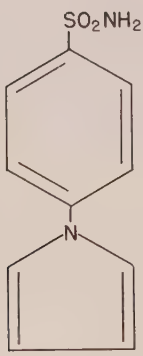
The seven compounds with three condensed rings (Fig. 1 and 2) are intensely interesting. In all of them the nitrogen occurs in the center one of the three rings. The nitrogen in four of them, carbazole, acridan, pheno-



2,5-Dimethylpyrrole



1,2-Bis(2,5-dimethyl-1-pyrryl)ethane



p-(1-Pyrryl)sulfonamide

FIGURE 3. N-substituted heterocycles.

thiazine, and phenothiazine-5-oxide, has an attached hydrogen, but the compound is inactive in spite of that. The nitrogen in acridine, phenazine, and phenazine oxide has no attached hydrogen, but the compounds are active. Something else must be involved in the condensed 3-ring compounds.

A more important structural difference between the inactive and the active type appears in the double bonds in the center ring. The double bonds are free to wander in the inactive compounds but they are fixed in an *o*-quinoid position for the active compounds (10).

EFFECT OF HYDROPHILIC SUBSTITUENTS

The classic substituents that confer fungitoxicity on organic molecules are reactive ones that tend to confer water solubility on them. This fact is

so obvious that it hardly warrants the space to say it. Water is an ubiquitous constituent of biological systems. It follows naturally from this that biological reactions must depend upon an aqueous medium. The fact that hydrophobic substituents may appear to confer toxicity is sometimes neglected. That action will be discussed in due course. The action of the hydrophilic substituents will be considered first.

EFFECT OF ACIDIC GROUPS

The two test organisms are well adapted to distinguish the action of acidic groups from other constituents of the molecule. Using cultures, Young and Bennett (31) have shown *Macrosporium* is an acidophobe; and as Cooley has shown (6) *Sclerotinia* is an acidophile. This is presumably due to the fact that *Macrosporium* lives in nature on the clover plant, which itself is an acidophobe; *Sclerotinia* attacks the acid fruit of peach, plum, or cherry.

On this basis, we think that it is fairly safe to conclude that if an acidic compound is more active against *Macrosporium* than against *Sclerotinia*, the action is probably due to the acidity. If other toxophores are also present, the compound may be toxic to *Sclerotinia* as well.

We have tested seven heterocyclic nitrogen compounds, containing sulfo or carboxylic groups. These are L-proline, *M*−, *S*−; *N*,*N*-1-tris(2-cyanoethyl)-L-histidine, *M*−,*S*−; 1-(2-cyanoethyl)-L-pyroglutamic acid, *M*+,*S*+; 8-quinolinol-5-sulfonic acid, *M*+,*S*−; quinolinic acid, *M*+, *S*−; quinolin-2-amic acid, *M*+,*S*− (all in Fig. 4). The seventh is 7-iodo-8-quinolinol-5-sulfonic acid, *M*+,*S*−. The results confirm the data for other nuclei with similar substituents. Only one is active on *Sclerotinia*. Five of the seven are active on *Macrosporium*. The two that are inactive on *Macrosporium* are amino acids or amino acid derivatives. In amino acids, of course, the acidic tendency of the carboxyl group is balanced by the basic tendency of the amino group. The pH therefore is not lowered to a level that is toxic to *Macrosporium*.

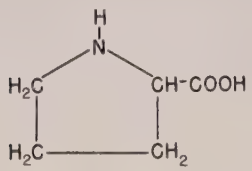
Later we shall be discussing the esters of a carboxyl derivative of pyridine, nicotinic acid.

A common property of many nitrogen compounds is to form salts with acids. Twenty-one of our heterocycles are so constructed (Table III and Fig. 5). Of these, nine are in the quinine group. As might be expected from the preceding discussion of acidic groups, all but two are toxic to *Macrosporium*. All but five are toxic to *Sclerotinia*, however, presumably because they contain other groups that are toxic to this organism. One, cinchonidine hydrochloride, is toxic to neither organism. These salts, except for effects on pH, would not be expected to behave differently from the parent compounds.

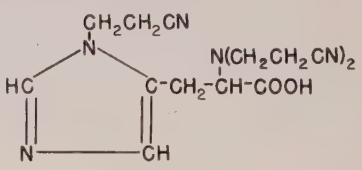
EFFECT OF HYDROXYL GROUPS

Knowledge of the effect of phenolic —OH on toxicity to microorganisms goes back to Lord Lister and his use of phenol in antiseptic surgery.

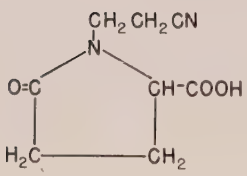
We have tested only three heterocycles with —OH substituents, quinoline, pyrimidine, and phenothiazine. Of these, only quinolinol was active.



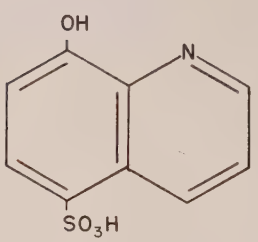
L-Proline



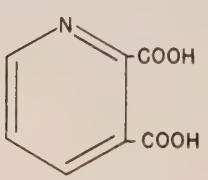
*N,N,*l-Tris(2-cyanoethyl)-L-histidine



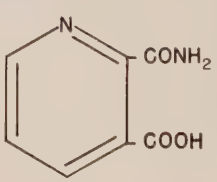
l-(2-Cyanoethyl)-L-pyroglutamic acid



8-Quinolinol-5-sulfonic acid



Quinolinic acid



Quinolin-2-amic acid

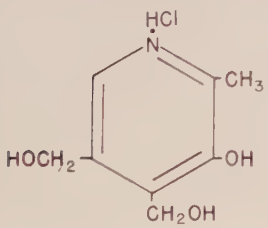
FIGURE 4. Acid substituted heterocycles.

The non-active pyrimidine derivatives (Fig. 6) were 5,6-dihydro-6-imino-5-isonitrosouracil, 5-amino-2-phenyl-2-*v*-triazolo[d]pyrimidine-7-ol, 2,6-diamino-5-nitroso-4-pyrimidol, 2,5,6-triamino-4-pyrimidol monosulfate monohydrate, 2,6-diamino-4-pyrimidol sulfate. The latter shows slight

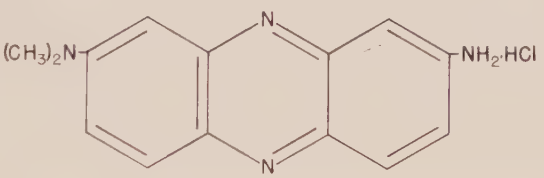
TABLE III

ACTIVITY OF SALTS FORMED FROM HETEROCYCLIC NITROGEN COMPOUNDS WITH ACIDS

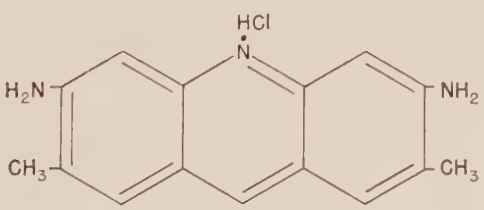
Compound	Toxicity		Illustration No.	
	M	*S*	Whole structure	Nucleus only
Pyridoxine hydrochloride	+	−	5	
Neutral red hydrochloride	+	+	5	
Acridine yellow hydrochloride	+	+	5	
Quinine hydrochloride	+	+	15	
Quinine sulfate	+	+		15
Quinine bisulfate	+	+		15
Quinine citrate	+	+		15
Quinine salicylate	+	+		15
Quinidine hydrochloride (isomer of quinine)	+	−		15
Cinchonine hydrochloride (quinine minus methoxy)	+	+		15
Cinchonine salicylate	−	+		15
Cinchonidine hydrochloride (isomer of cinchonine)	−	−		15
8-Quinolinol sulfate	+	+		1
8-Quinolinol benzoate	+	+		1
2,6-Diamino-4-pyrimidol sulfate	+	−	6	
2,5,6-Triamino-4-pyrimidol monosulfate	+	−		6
3-(2-Piperidino-1-hydroxy-*n*-propyl)phenanthrene hydrochloride	+	+		14
3-(3-Pipiridino-1-oxo-propyl)phenanthrene hydrochloride	+	+	14	
2-[3-(1,2,3,4-Tetrahydroisoquinolino)-1-oxo-propyl-phenanthrene hydrochloride	+	+	14	
2-[3-(1,2,3,4-Tetrahydroisoquinolino)-1-hydroxy-*m*-propyl]phenanthrene hydrochloride	+	+		14
3-[(1,2,3,4-Tetrahydroisoquinolino)methyl]-4-hydroxy-1,2,3,4-tetrahydrophenanthrene hydrochloride	+	+	14	



Pyridoxine hydrochloride



Neutral Red



Acridine Yellow

FIGURE 5. Acid salts of compounds containing nitrogen.

toxicity to *Macrosporium*, but this is almost surely due to the acidity to which *Macrosporium* is very sensitive.

3-Hydroxyphenothiazine and thionol (7-hydroxyphenothiazone) (Fig. 6) were not active against either fungus. Abiusso (1) reported toxicity of the latter compounds to *Gloeosporium* and *Fusarium*. 1-Methyl-3-hydroxyphenothiazine and 7-ethoxy-3-hydroxyphenothiazine show very slight toxicity to *Sclerotinia* only. This is probably an action of the aliphatic substituents as will be discussed below.

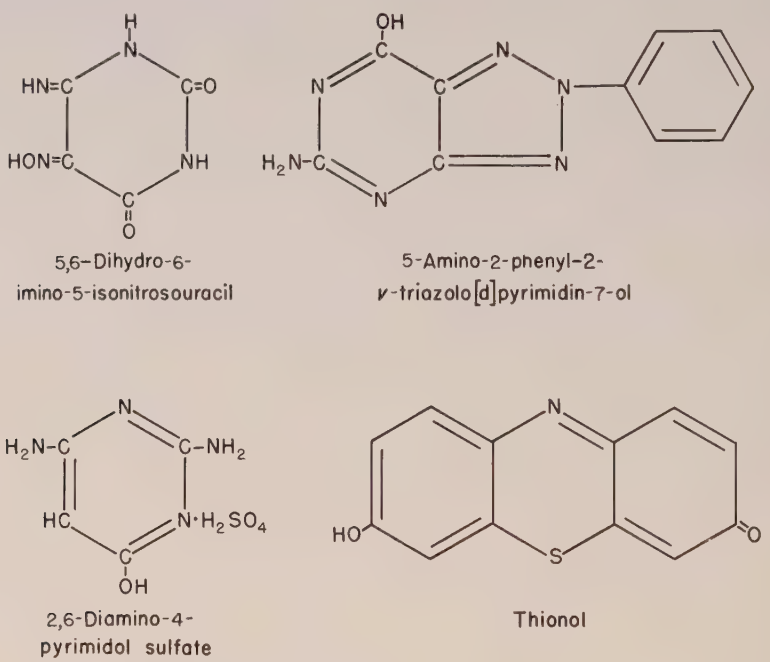


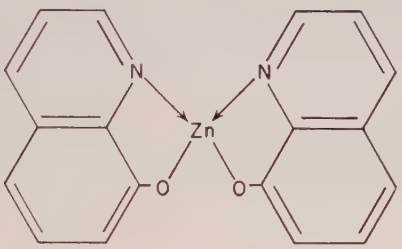
FIGURE 6. Hydroxyl substituted heterocycles.

The fungitoxicity of the quinolinols (see Fig. 1) has occupied the attention of this laboratory ever since we began to use them in the chemotherapy of Dutch elm disease in 1941 (34), and since Zentmyer (32) made the significant suggestion in 1943 that the action of 8-quinolinol is due to its probable ability to chelate the essential metals of the fungus (zinc salt, illustrated in Fig. 7). This action of 8-quinolinol was discovered, apparently independently, but somewhat later by Albert *et al.* (2) in Australia who were working with bacteria.

The effect of substituting a sulfo group in the 5-position (Fig. 4) is interesting and critical to a discussion of mechanism. The sulfo group completely quenches the toxicity to *Sclerotinia* of 8-quinolinol and of 7-iodo-8-quinolinol. It does *not* quench the toxicity to *Macrosporium*.

Let us consider first the difference between the organisms. 8-Quinolinol-5-sulfonic acid is toxic to *Macrosporium*, not to *Sclerotinia*. Considering this isolated fact in the light of data given above on acid substituents, we have no trouble in concluding that the acidic grouping confers the toxicity on *Macrosporium*. The second fact to be worried about is that 8-quinolinol is toxic to *Sclerotinia*, whereas 8-quinolinol-5-sulfonic acid is not. Let us accept the reasonable deduction that 8-quinolinol owes its fungitoxicity to its ability to chelate the metals that the fungus requires. If so, putting a sulfo grouping in the 5-position must destroy its ability to chelate metals by the mechanism of the parent compound. Albert *et al.* (2) have determined this to be so.

Putting these two deductions together we conclude that 8-quinolinol



8-Quinolinol, zinc salt

FIGURE 7. Zinc-8-hydroxyquinolinate.

chelates the metals of both spores and is toxic. 8-Quinolinol-5-sulfonic acid does not chelate metals and is not toxic by that mechanism to either spore. Its acidic property, however, makes it toxic to *Macrosporium*.

The quenching action of the sulfo grouping must be to act competitively with the —OH grouping in the molecule. This is the paradox of the hungry mule positioned halfway between two equally attractive piles of hay—he starves to death. The experimental approach to this paradox is to make one pile of hay inedible. The mule eats the other. In our case, we inactivated the sulfo grouping with zinc as in 8-quinolinol-5-sulfonic acid, zinc salt. The toxicity to *Sclerotinia* was restored. Albert *et al.* (2) made an ester of the sulfonic acid and restored the lost toxicity. This result leads one to think that the acidic group reduces the pH of the spore suspension to a point where the 8-quinolinol does not chelate readily (see Zentmyer, 33).

This is probably not the whole story, however. Mason (20) reports that nitro groups in the 5- and 7-positions quench the potency of 8-quinolinol to *both Sclerotinia* and *Macrosporium*. This observation is useful to this discussion. It almost clinches the explanation for the toxicity of the 5-sulfonic acid compound to *Macrosporium*. The nitro group is just as effec-

tive as the sulfo group in quenching the toxicity. Only the sulfonic acid restores toxicity to *Macrosporium*. Hence, it must be the acid.

However, the nitro group would not reduce the pH to a level where chelation would fail to occur. Whether 5,7-dinitro-8-quinolinol would chelate metals, we do not yet know. The data do fit another hypothesis of fungitoxic action. Horsfall and Rich (12) have shown that oxidized substituents often quench the toxicity of reduced substituents. Their thesis is that the fungus depends for its livelihood on its ability to oxidize its substrate to obtain energy. The fungus may very well oxidize to its own detriment the reduced compound instead of the normal substrate.

The action of 2-quinolinol should be interesting. Albert *et al.* (2) found that it is incapable of forming chelate complexes with metal. Under the chelation theory, it should not be toxic. They reported that it is not. Rigler and Greathouse (25) say that it is only 1/600 as toxic to the *Phymatotrichum* fungus as 8-quinolinol. In our tests it was weakly toxic to only one of our organisms, *Macrosporium*.

Most 8-quinolinol compounds have peculiar dosage-response curves as mentioned briefly by Barratt and Horsfall (4). The curves are bimodal. There are two peaks. That means that between the two peaks toxicity increases as concentration decreases. This is easy to write off as experimental error, but it definitely is not.

No very certain explanation for this phenomenon exists. Barratt and Horsfall (4) give numerous instances and extensively discuss the general problem. They conclude that a polymodal curve indicates that the compound is acting by more than one mechanism. This suggests that 8-quinolinol acts by at least two mechanisms.

The chelation theory provides for only one mechanism. Possibly metal salts of 8-quinolinol act by the second mechanism. If true, then metal salts should not show double curves. Double curves do not occur in the data for two tests with 8-quinolinol, calcium salt, and one test with 8-quinolinol, magnesium salt. Mason (20), however, reports double curves for metal salts

Substitution of methyl in the 4-position of 2-quinolinol added no potency to 2-quinolinol.

The copper complex of 8-quinolinol has recently been revived by Powell (23) as a foliage fungicide. This was used earlier in France. Several investigators, notably Ciferri and Baldacci (5), Mason and Powell (21), and lately Manten, Klöpping, and van der Kerk (18) have called Zentmyer's theory into question on the basis of the fact that the copper complex is fungitoxic. The essence of their argument is this: 8-quinolinol should not be able to chelate with the metals in the spore if it is tied up in a chelated complex first.

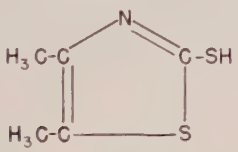
Vicklund and Manowitz (28) have attacked this conclusion and supported Zentmyer (33). They have shown that the preformed copper complex ionizes to some extent, thus liberating copper ions and 8-quinolinol

ions. They find that the fungus secretes an enzyme that tends to destroy 8-quinolinol. This enzyme action is poisoned by copper. Hence, the copper salt is the most active of the 8-quinolinols because it has a double action. The copper prevents the destruction of the 8-quinolinol which in turn inhibits the spore by combining with the needed metals. The 8-quinolinol problem does not yet appear to be completely solved, but it is a good example of multiple action of fungicides.

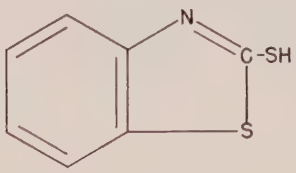
As shown above, the amino acid, L-proline, is not active. If, however, an —OH is inserted in the 4-position, the compound becomes fungitoxic as Robbins and McVeigh (26) have shown. They suggest that the compound may interfere with the nitrogen metabolism of the cell.

EFFECT OF SULFHYDRYL GROUPS

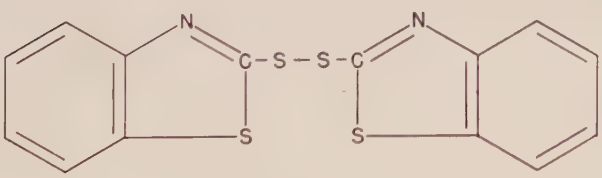
—SH is not as old a toxophore historically as —OH, but it is just as important, especially to fungi. We have data (Fig. 8) on only four thiol com-



4,5-Dimethyl-2-thiazolethiol



2-Mercaptobenzothiazole



2,2'-Dithiobis(benzothiazole)

FIGURE 8. Thiol substituted heterocycles.

pounds, 4,5-dimethyl-2-thiazolethiol, 2-mercaptobenzothiazole (Captax), 2-mercaptobenzothiazole, zinc salt, and 2,2′-dithiobis(benzothiazole). Both thiazolethiols were reasonably fungitoxic to both organisms. Marsh (19) appears to have been the first to try 2-mercaptobenzothiazole. Everitt and Sullivan (7) report that 2-phenylbenzothiazole was toxic to various fungi in culture, but not so toxic as 2-mercaptobenzothiazole. We have a fair amount of additional data on 2-mercaptobenzothiazole as a fungicide. In

eight tests of the compound the mean ED50 on *Macrosporium* was 8.4 μg. per cm.^{2} and a dosage-response slope was 4.60.

The —SH group clearly is essential to toxicity since (a) neither benzothiazole (Fig. 1) nor 2-chlorobenzothiazole was fungitoxic; (b) if two molecules of 2-mercaptobenzothiazole are combined at the —SH groups to give an —S—S— linkage [2,2′-dithiobis(benzothiazole)] (Fig. 8), the toxicity is quenched.

Albert *et al.* (2) suggest that the bactericidal action of 2-mercaptobenzothiazole is due also to its ability to chelate metals. The opponents of the chelation theory might well bring to their support the fact that the zinc salt of 2-mercaptobenzothiazole is toxic to fungi just as the metal salt of 8-quinolinol is toxic. The zinc salt of 2-mercaptobenzothiazole in a single experiment had an ED50 of 8.0 μg./cm.^{2} and a dosage-response slope of 4.80. The slope is the same as that for the parent compound, indicating that the mode of action is the same. The fact that the ED50 is the same suggests, however, that the zinc salt is weaker, because it contains two moieties of benzothiazole.

One is tempted to speculate on the basis of the chelation theory that useful toxicants could be prepared from other heterocycles by designing them with an —OH or —SH or a carbon atom, adjacent to the nitrogen. Albert *et al.* (3) have already shown that 1-hydroxyacridine is active.

EFFECT OF A CARBONYL GROUP IN THE HETEROCYCLE

The carbonyl group is one that often confers fungitoxicity on a molecule especially if the carbon occurs in a ring. Numerous heterocycles with carbonyl groups in the ring have been examined.

Inspection of the data (Tables IV and V) shows that single carbonyls are less likely to impart fungitoxicity than double carbonyls. Twelve compounds with a single C=O or C=S in the ring were tested (Table IV and Fig. 9). Six showed some toxicity to *Macrosporium*, five to *Sclerotinia*, four to both organisms.

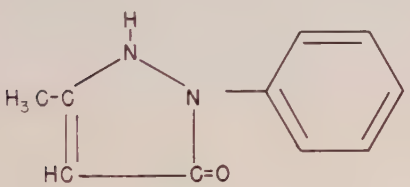
1-(2-Cyanoethyl)—L—pyroglutamic acid, was toxic to *Macrosporium* and not to *Sclerotinia*. As we have already suggested, however, this is probably due to the —COOH group, not to the carbonyl. Of the five pyrazolones, two were toxic, one (picrolonic acid) undoubtedly because of the —NO_2 groups. The other, 5-methyl-2-phenyl-3-pyrazolone, is not active against *Macrosporium*, only very weak against *Sclerotinia*. It has hydrogen attached to the nitrogen and is thus reminiscent of other toxic heterocycles so constructed.

o-Benzoic sulfimide (saccharin) is toxic to *Macrosporium*, not toxic to *Sclerotinia*. The reason for this is not clear, but it is interesting that conversion to the sodium salt quenches the activity. We suspect that this is due to the fact that the sodium salt ionizes sufficiently to inhibit permeation.

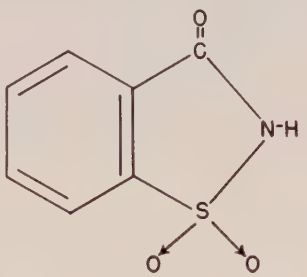
TABLE IV

ACTIVITY OF COMPOUNDS WITH A SINGLE C=O OR C=S GROUP IN THE RING

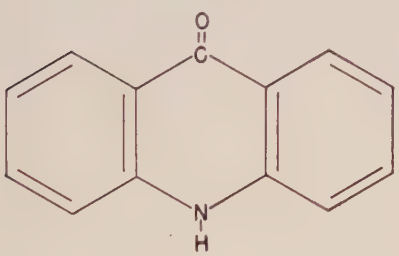
Compound	Toxicity		Illustration No.	
	M	*S*	Whole structure	Nucleus only
1-(2-Cyanoethyl)-L-pyroglutamic acid	+	−	4	
o-Benzoic sulfimide (saccharin)	+	−	9	
o-Benzoic sulfimide, sodium salt	−	−		9
1,4-Dihydro-1-methyl-4-quinolinethione	+	±	9	
Acridone	+	+	9	
Phenothiazone	+	+		6
Thionol	−	−		
5-Methyl-2-phenyl-3-pyrazolone	−	±	9	
2-Phenyl-1,5-dimethylpyrazolone	−	−		9
4-Amino-2-phenyl-1,5-dimethylpyrazolone	−	−		9
N-[β-(4-Antipyrylamino)crotonyl]aniline	−	−	11	
Picrolonic acid	+	+	13	



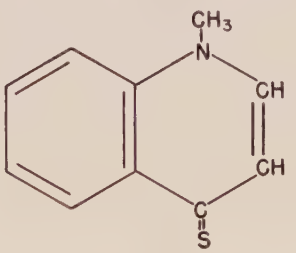
5-Methyl-2-phenyl-3-pyrazolone



o-Benzoic sulfimide



Acridone



1,4-Dihydro-1-methyl-4-quinolinethione

FIGURE 9. Single substitution of C=O and C=S.

This does not prove, in other words, that the hydrogen on the nitrogen is inactive. It would have to be substituted to prove that.

Phenothiazone, acridone, and 1,4-dihydro-1-methyl-4-quinolinethione are quinoid structures based on carbonyl or C=S and are presumably toxic on that account. The action of acridone provides some evidence for

this. If the carbonyl group in acridone is reduced as in acridan (Fig. 1), the activity is lost. Goldsworthy and Green (9) first reported the fungitoxicity of phenothiazone.

Thionol is a strange compound. It may be written as 7-hydroxyphenothiazone. If so, it is a quinoid structure that is non-active. Perhaps this is another case of interference between oxidized and reduced toxophores.

The heterocycles containing two carbonyls in the ring are especially interesting to us. Many of them are quite fungitoxic. The first one that came to significant notice is actidione (Table V and Fig. 10), which is a

TABLE V

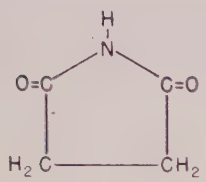
ACTIVITY OF COMPOUNDS WITH TWO C=O OR C=S GROUPS IN THE RING

Compound	Toxicity		Illustration No.	
	M	*S*	Whole structure	Nucleus only
1-Acetyl-2-thiohydantoin	+	+		
Succinimide	−	−	10	
Phthalimide	+	±	10	
Quinolinimide	+	±	10	
"Orthocide 406"; (*N*-(Trichloromethylthio)-1,2,3,6-tetrahydrophthalimide)	+	+	10	
Actidione	+	+	10	
Thiobarbituric acid	+	+	10	
5-(*p*-Dimethylaminobenzylidene)rhodanine	+	+	10	
6-Aminouracil	−	−	12	
3-Aminophthalhydrazide	−	−	10	

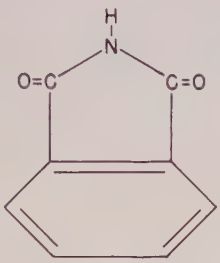
by-product of streptomycin manufacture. Its fungitoxicity was first reported by Whiffen *et al.* (30). The second one is "Orthocide 406", *N*-(trichloromethylthio)-1,2,3,6-tetrahydrophthalimide. These both have the two carbonyls separated by —NH— in the ring. These two compounds are as fungitoxic as any discussed herein. Another powerful fungicide of this type is *N*-nitrosophthalimidine (15).

Data on these related compounds are given in Table V. Six are toxic to both organisms. Thiobarbituric acid is almost as toxic to *Macrosporium* as actidione or "Orthocide 406." Thiobarbituric acid is not strictly in the group because it has one C=S instead of two carbonyls. 5-(*p*-Dimethylaminobenzylidine)rhodanine also has C=S replacing one of the carbonyl groups, but the compound is fungitoxic as expected from the rest. In fact, Horsfall and Zentmyer (13) published on its fungitoxicity in 1944, in connection with their studies of metal reagents as fungicides. Albert *et al.* (2) tested it independently as a bactericide. They say that it acts to chelate metals. To that extent its activity is in accord with that of 8-quinolinol.

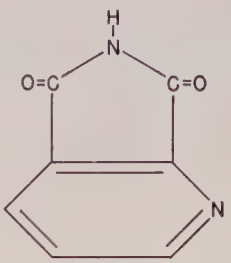
The performance of the $\begin{matrix} O & H & O \\ \| & | & \| \\ -C- & N & -C- \end{matrix}$ structure in many types of rings



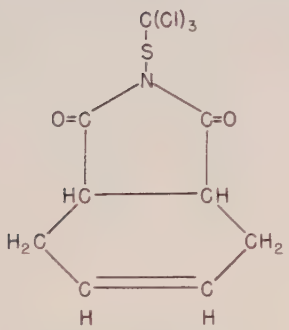
Succinimide



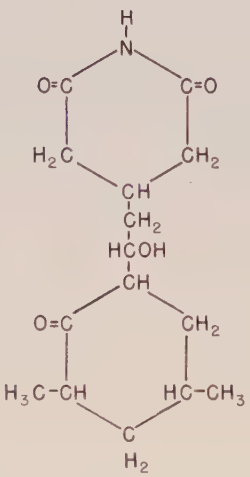
Phthalimide



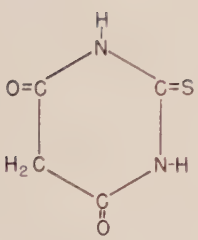
Quinolinimide



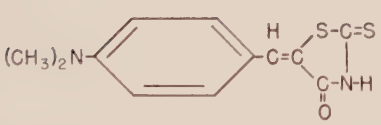
N-(Trichloromethylthio)-
1,2,3,6-tetrahydrophthalimide
"Orthocide 406"
"SR 406"



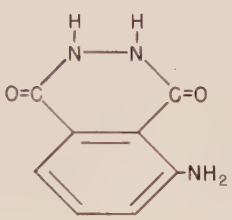
Actidione



Thiobarbituric acid



5-(*p*-Dimethylaminobenzylidene)-
rhodanine



3-Aminophthalhydrazide

FIGURE 10. Double substitution of C=O or C=S.

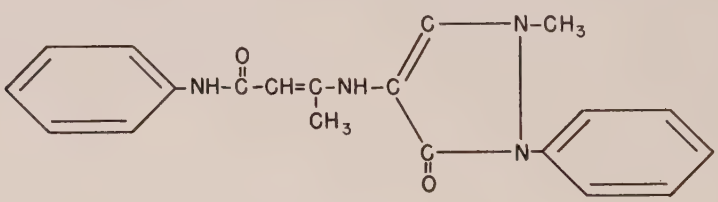
suggests that it is a strong toxiphore. Succinimide is an exception. It is not active. Whether this is due to low toxicity or to low permeation is not clear, but we suggest that it is probably poor permeation because it does not contain a lipophilic group.

There are two other exceptions to the generalization that two carbonyl groups in a heterocyclic nitrogen compound impart fungitoxicity. These,

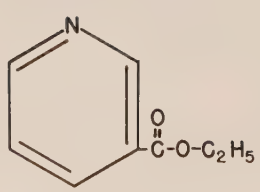
TABLE VI

ACTIVITY OF COMPOUNDS WITH A C=O GROUP IN A SIDE CHAIN

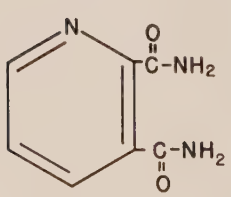
Compound	Toxicity		Illustration No.	
	M	*S*	Whole structure	Nucleus only
N-[β-(4-Antipyrylamino)crotonyl]aniline	−	−	11	
Quinolinamide	−	−	11	
Quinolinic acid, methyl ester	−	−	11	
N-(1-Oxolauryl)phenothiazine	−	−		1
10-Benzoyl-3,7-bis(dimethylamino)phenothiazine	−	−		1
3-(3-Piperidino-1-oxo-propyl)phenanthrene hydrochloride	+	+	14	
2-[3-(1,2,3,4-Tetrahydroisoquinolino)-1-oxo-propyl]phenanthrene hydrochloride	+	+	14	
Nicotinic acid esters (See Table IX)	−	−	11	



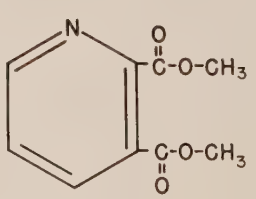
N-[β-(4-Antipyrylamino)crotonyl]aniline



Nicotinic acid, ethyl ester



Quinolinamide



Quinolinic acid, methyl ester

FIGURE 11. C=O as part of a side group.

listed at the bottom of Table V, are 6-aminouracil and 3-aminophthalhydrazide. It is probably significant that both molecules contain amino groups in addition to the carbonyls. Hence, the molecule contains both an oxidized and a reduced group. Horsfall and Rich (12) have shown that other such compounds are inactive.

EFFECT OF CARBONYL GROUPS IN THE SUBSTITUENT

Sometimes in this paper we have decided that a structure is a good toxophore on the basis of proportion of successful molecules that contain it. On that basis, we must conclude that carbonyl in a substituent group attached to a heterocyclic nitrogen compound does not lend strong toxicity. Of the 14 compounds tested (Tables VI and IX, and Fig. 11) seven were appreciably toxic to *Sclerotinia*. Two were toxic to *Macrosporium* unless they were acid salts as in the two phenanthrene compounds. Horsfall, Chapman, and Rich have shown elsewhere (11) that the C=O group is more toxic to *Sclerotinia* than to *Macrosporium*. This is surely true here.

EFFECT OF AMINO GROUPS

$—NH_2$ was ineffective as a means of making heterocyclic compounds fungitoxic. Twelve compounds were assayed (Table VII and Fig. 12). Only

TABLE VII

ACTIVITY OF $—NH_2$ ATTACHED TO THE RING

Compound	Toxicity		Illustration No.	
	M	*S*	Whole structure	Nucleus only
2,6-Diamino-5-nitroso-4-pyrimidol	−	−		
2,6-Diamino-4-pyrimidol sulfate	+	−	6	
2,5,6-Triamino-4-pyrimidol monosulfate	±	−		6
6-Aminouracil	−	−	12	
3-Aminophthalhydrazide	−	−	10	
2-Aminopyridine	−	−		1
Thionine (3,7-diaminophenothiazine)	+	+		1
Acridine yellow hydrochloride	+	+	5	
Neutral red hydrochloride	+	+	5	
2-Aminothiazole	±	±	12	
2-Aminonaphtho(2,1)thiazole	−	±	12	
2-Amino-4-(*p*-biphenylyl)thiazole	−	±	12	
5-Amino-2-phenyl-2-*v*-triazolo[d]-pyrimidin-7-ol	−	−	6	

one, other than the three dyes, showed any significant activity. This result contrasts sharply with the effect of $—NH_2$ attached to a diphenyl nucleus. There, $—NH_2$ often confers toxicity (11). On the basis of performance of carbonyl in a heterocycle, one would expect that 6-aminouracil (Fig. 12) might be active. Since it is not, then the amino group must be quenching the toxicity as discussed above under the action of the carbonyl group.

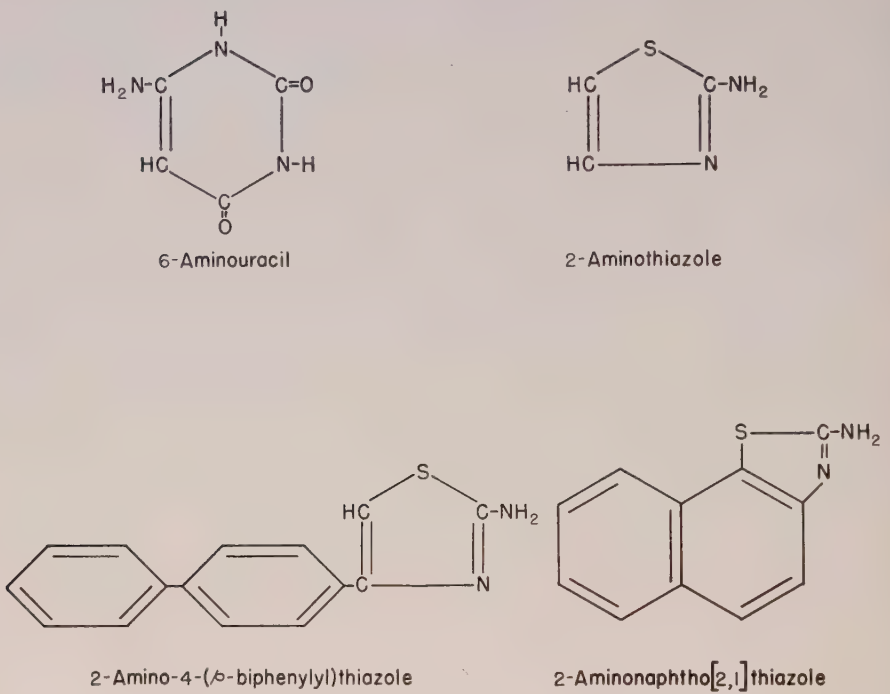


FIGURE 12. Amino substituted heterocycles.

EFFECT OF NITRO GROUPS

The nitro (—NO_2) group is usually a powerful activating group on fungitoxicity. It was no exception here. It activates antipyrene (see picrolonic acid,[1] Fig. 13), quinoline, chloroquinoline, and chlorobenzothiazole. 2-Chloro-x-nitrobenzothiazole must act by a different mechanisn from 2-mercaptobenzothiazole because the slope of the dosage-response curve was 2.2 as compared with 4.6 for 2-mercaptobenzothiazole.

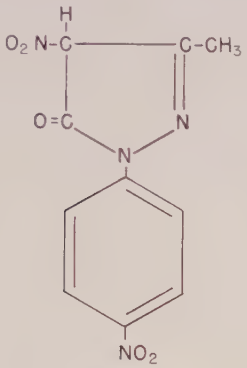
Mason and Powell (21) have shown, however, that nitro groups in the 5- and 7-positions of 8-quinolinol suppress the potency. This is very interesting in the light of the fact that sulfo and nitro groups act similarly in some cases. It will be remembered that a sulfo group in the 5-position also quenches the toxicity of 8-quinolinol.

Oster and Golden (22) have shown that nitration of quinoline (not quinolinol) in the 6- or 8-position makes it fungitoxic. This is probably on account of oxidation.

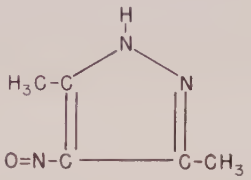
EFFECT OF NITROSO GROUPS

Likewise, —N = O is a powerful toxophore. McNew and Sundholm (17) showed that various derivatives of 4-nitrosopyrazole (Fig. 13) are powerful

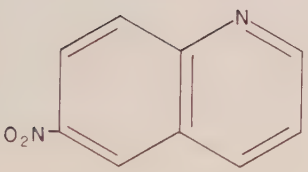
fungitoxicants. We used several of these in testing the action of lipophilic groups on activity. The activities are discussed below. Ladd (15) has patented *N*-nitrosophthalimidine as a fungicide. One of our nitroso compounds was essentially inactive, 2,6-diamino-5-nitroso-4-pyrimidol. Perhaps, the $—NH_2$ or the —OH inhibited the usual fungitoxic action of the nitroso group.



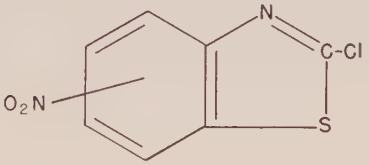
Picrolonic acid



3,5-Dimethyl-4-nitrosopyrazole



6-Nitroquinoline



2-Chloro-x-nitrobenzothiazole

FIGURE 13. Nitro and nitroso substituted heterocycles.

EFFECT OF QUATERNARY NITROGEN

The fungitoxic action of quaternary ammonium derivatives is so well known as hardly to be worth discussion. This applies whether the nitrogen occurs in a ring or not. Howard *et al.* (14) have reported the fungitoxicity of pyridinium, quinaldinium, lutidinium, picolinium, and isoquinolinium compounds. Lauryl isoquinolinium bromide (16) had a brief flurry as a field fungicide for apple scab. It has topical chemotherapeutic value, but it lacked sufficient protective value to make it successful.

EFFECT OF HALOGENATION

Chemists often chlorinate organic compounds to enhance their reac-

tivity. Often this procedure does not produce the desired effect on the activity toward fungi (10). In many cases, the opposite effect is to be noted.

We tested, for example, various chlorinated 2-quinolinols: 4-chloromethyl-2-quinolinol, 8-chloro-4-methyl-2-quinolinol, and 8-chloro-4-chloromethyl-2-quinolinol. In all cases the toxicity was quenched. Similarly, Mason and Powell (21) showed that substitution of chlorine or bromine in the 5- and 7-positions reduced the fungitoxicity of both 8-quinolinol and 8-quinolinol, copper salt. Oster and Golden (22) similarly showed that 5,7-dibromo-8-quinolinol and 5-chloro-7-iodo-8-quinolinol were less active against a human pathogenic fungus—*Trichophyton*.

On the other hand, chlorine in the 7-position clearly enhanced the activity of 2,2,4-trimethyl-1,2-dihydroquinoline to *Sclerotinia*. This is evidence that dihydroquinoline and 8-quinolinol have different modes of action.

EFFECT OF LIPOPHILIC SUBSTITUENTS

One of the most interesting outcomes of this study of the activity of heterocyclic nitrogen compounds was the apparent significance of aliphatic and aromatic substituents on the heterocycle.

It is not difficult to see how hydrophilic groups that are chemically reactive can make a nucleus fungitoxic. They give it opportunity to react.

Of much more interest is, what function can a hydrocarbon group perform? It is usually chemically inert. Hydrocarbon substituents are notably effective in making compounds fungitoxic. One of the most striking in our experience is the action of a 2-hendecyl group on 2-oxazoline. Horsfall (10) reported that 2,4,4-trimethyl-2-oxazoline (Fig. 1) was not fungitoxic. 2-Hendecyl-2-oxazoline, on the other hand, is a powerful spore inhibitor. Rich and Horsfall (24) are proposing elsewhere that the hydrocarbon groups act through their lipophilic properties to increase permeation of the compound into the cell. They do not increase toxicity. They increase activity. A 2-hendecyl group does not make 2-oxazoline toxic. It was toxic all the time. It was not able to permeate into the cell. It could not exert its toxicity on the outside of the cell.

We think that the increase in permeability arises because the hydrocarbon groups enable the compound to dissolve—not in the water that bathes the cell, but in the fats of the semipermeable membrane that encloses the cell. This obviously is in accordance with the Overton-Meyer theory of permeability.

The discovery of the activating effect of hydrocarbon groups is old. Treat B. Johnson, Yale Professor of Chemistry, added greatly to his wealth by patenting hexylresorcinol. He did not discover the bactericidal effect of resorcinol, just the patentable activating effect of the hexyl group.

Perhaps the first example of an activated heterocycle that came to our

attention was 3-phenylaminophenothiazine and 3-(*p*-ethoxyphenylamino)-phenothiazine. These were both found to be toxic to our test organisms in late 1942. Since phenothiazine (Fig. 1) is not active, it is clear that these two groups imparted activity. One might assume that the toxicity lies in the —NH— bridge. Diphenyl amine is not toxic, however (11); in fact, few secondary amines are fungitoxic. The hydrocarbon ring must be the activating group, since it is almost as effective as the ethoxyphenyl group.

Our data on most heterocyclic nuclei are only tantalizingly suggestive.

TABLE VIII

EFFECT OF LIPOPHILIC GROUPS ON ACTIVITY OF 6-ATOM HETEROCYCLIC NITROGEN COMPOUNDS

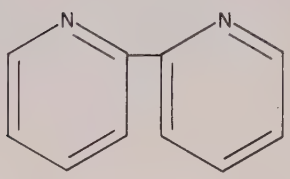
Compound	Toxicity		Illustration No.	
	M	*S*	Whole structure	Nucleus only
1-Methylpryidine	−	−		1
3-Methylpyridine	−	−		1
3,4-Dimethylpyridine	−	−		1
2,6-Dimethylpyridine	−	−		1
1,4,6-Trimethylpyridine	−	−		1
2-(*n*-Amyl)pyridine	−	−		1
4-(*n*-Amyl)pyridine	−	±		1
Sulfapyridine, sodium salt	−	−		1
2-Stilbazole	+	+	14	
2,6-Distyrylpyridine	−	−	14	
α,α'-Dipyridyl	−	+	14	
3-(2-Piperidino-1-hydroxypropyl)-phenanthrene hydrochloride	+	+		14
3-(3-Piperidino-1-oxopropyl)-phenanthrene hydrochloride	+	+	14	
Actidione	+	+	10	

We are convinced, however, that many of the heterocycles could be persuaded to kill fungi by the addition of the proper hydrocarbon at the proper place.

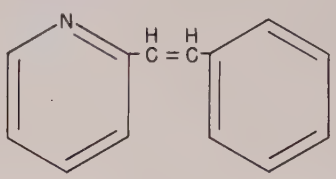
Data on nitrosopyrazole, imidazoline, and esters of nicotinic acid will be presented below in terms of the effect of chain length and position.

We have studied 14 piperidine and pyridine (Fig. 1) heterocycles with hydrocarbon substituents (Table VIII and Fig. 14). Two are piperidines (reduced pyridine) and the others are pyridines.

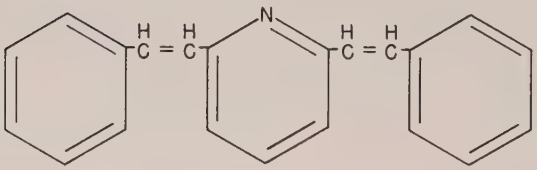
Methylation in any single, double or triple position on pyridine was incapable of adding toxicity. Similarly, 2-(*n*-amyl)pyridine was not toxic, but 4-(*n*-amyl)pyridine was weakly toxic to *Sclerotinia*. The sodium salt of sulfapyridine was non-toxic, but it is not strictly germane here because it is water soluble. 2-Stilbazole was very active against *Sclerotinia* but somewhat less active against *Macrosporium*. 2-Stilbazole is pyridine to which styrene is attached in the 2-position. This hydrocarbon group makes



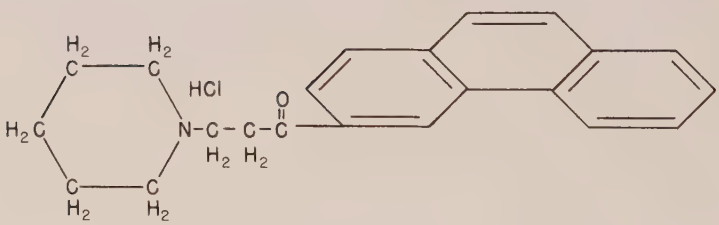
α,α′-Dipyridyl



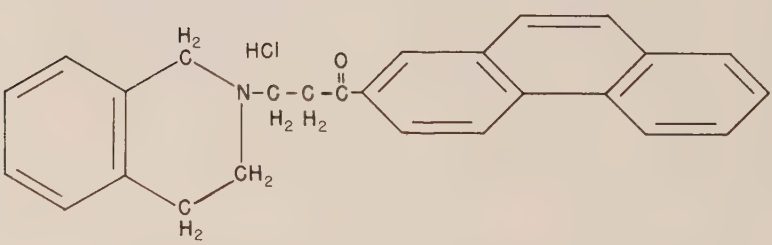
2-Stilbazole



2,6-Distyrylpyridine



3-(3-Piperidino-1-oxopropyl)phenanthrene hydrochloride

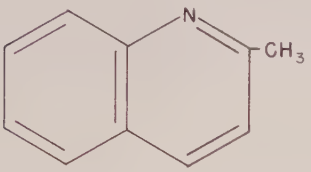


2-[3-(1,2,3,4-Tetrahydroisoquinolino)-1-oxopropyl]phenanthrene hydrochloride

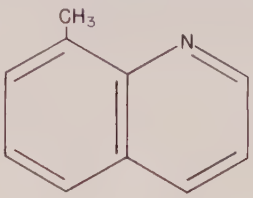
FIGURE 14. Lipophilic substituted heterocycles.

pyridine significantly fungitoxic to both organisms. One might suspect the double bond of the styrene moiety as the toxophore, were it not that the same grouping repeated in the 6-position is not active (2,6-distyrylpyridine). It may be significant, however, that α,α'-dipyridyl is only weakly fungitoxic. Even though one pyridine nucleus may be considered as a lipophilic group on the other, the compound is still essentially inactive.

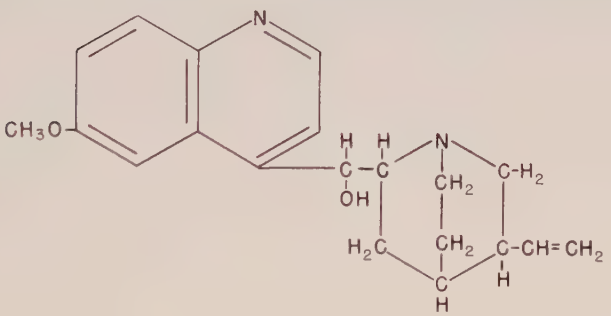
It is interesting that of the three pyridine compounds so far shown as



2-Methylquinoline



8-Methylquinoline



Quinine

FIGURE 15. Lipophilic substituted heterocycles.

toxic, all are more toxic to *Sclerotinia* than to *Macrosporium*. We have data on two piperidine compounds (Fig. 14) each of which has a phenanthrene group attached through a propane bridge. Both were reasonably toxic. Since both are hydrochlorides, we cannot be sure whether they, like pyridine, are more toxic to *Sclerotinia* than to *Macrosporium* or not. HCl increases the toxicity to *Macrosporium*, as we have already seen.

Similarly, we have data on a few quinoline compounds substituted with hydrocarbon groups. Quinoline itself (Fig. 1), as mentioned above, is not active. Quinaldine (Fig. 15) is slightly active against both organisms, especially *Sclerotinia*. This is reminiscent of the situation with pyridine. 8-Methylquinoline (Fig. 15) was not active.

Rigler and Greathouse (25), growing the fungus *Phymatotrichum omni-*

vorum in culture, reported as follows on the fungitoxicity of methylquinolines: 6-methylquinoline was the most potent of the single substitutions followed in decreasing order by 4-, 7-, 2-, and 8-methylquinoline. The 2,6-dimethylquinoline was more inhibitory than the 2,4-dimethyl compound. The total evidence, then, indicates that a hydrocarbon substituent in the 6-position has greater potency than one in the 4-position.

One wishes for a quinoline with a big lipophilic attachment, preferably in the 6-position, perhaps a 17-carbon chain, or a styrene group. A few of the compounds that we have, may perhaps throw light on the likelihood that this wish could be fulfilled. Quinine and cinchonine (Table III and Fig. 15) perhaps will suffice for this need just now. They may be considered as quinoline with a large lipophilic substituent in the 4-position which is next to the 6-position in importance. Both are toxic to both organisms. These two differ only in that quinine has a methoxy group in the 6-position on the quinoline nucleus, cinchonine does not. Unfortunately, we do not have sufficient data to distinguish the significance of the 6-methoxy group in quinine. Perhaps acridine also (Fig. 2) could be examined in this connection. It could be considered as quinoline with a hydrocarbon group condensed at the 2- and 3-positions. If so, it serves to make quinoline very active.

As in the case of the piperidine nucleus (Table III), the attachment of phenanthrene may have helped to account for the fungitoxicity of the tetrahydroisoquinoline nucleus. This, however, is uncertain, because we had no tetrahydroisoquinoline with which to compare it.

It seems significant that acridan (Fig. 1) is essentially not active, but 9,9-dimethylacridan is toxic to *Sclerotinia*. Also 3-hydroxyphenothiazine is not active but 1-methyl-3-hydroxyphenothiazine is slightly active against *Sclerotinia*. We have alluded above to the action of 3-phenylamino in imparting toxicity to phenothiazine. We should like to test phenothiazine compounds with other hydrocarbon substituents in the 1-position or possibly other positions.

EFFECT OF CHAIN LENGTH

We have a fairly complete series of hydrocarbon esters of nicotinic acid (Table IX). It is noteworthy that the ethyl ester is not active. It would be easy to say—not toxic. The activity for *Macrosporium* increases as the chain lengthens up to 6 and then decreases sharply. The activity for *Sclerotinia* increases up to 8 and then declines sharply. It is of further interest that cyclohexyl is a more powerful activator than normal hexyl for either organism.

Wellman and McCallan (29) discovered the strong activating effect of the heptadecyl group in the 2-position on 2-imidazoline (glyoxalidine) or 1-hydroxyethyl-2-imidazoline. They showed an "increase in fungistatic

action as the number of carbon atoms in the aliphatic chain in the 2-position is increased to a maximum in the vicinity of 17 carbon atoms beyond which it falls off" (29, p. 152). They were somewhat concerned as to the significance of the effect. They say (p. 153), "A logical assumption for this maximum in fungistatic action would be that there is continually increasing toxicity in the series until the point where decreasing solubility allows less than a toxic concentration in solution." This they could not confirm, of course. They were concerning themselves with water solubility, not lipoid solubility.

They attempted to measure absorbability of the compound by agitat-

TABLE IX

FUNGITOXICITY OF NICOTINIC ACID ESTERS, AND OF 2-IMIDAZOLINE HOMOLOGS. DATA EXPRESSED AS PERCENTAGE OF SPORES NOT GERMINATING

Name of substituent	No. of carbons	Micrograms per cm.²							
		Macrosporium				*Sclerotinia*			
		1300	130	13	1.3	1300	130	13	1.3
Data for esters of nicotinic acid									
Ethyl	2	0	0	0	0	0	0	0	0
Butyl	4	100	13	0	0	100	91	0	0
Amyl	5	100	63	0	0	100	100	0	0
Hexyl	6	77	10	9	0	100	62	18	0
Cyclohexyl	6	100	53	6	4	100	100	6	0
Octyl	8	12	7	1	0	100	100	100	0
Decyl	10	4	4	0	0	23	0	2	0
Data for substituents in 2-position on 2-imidazoline									
Methyl	1	100	Ca 50	0	0	100	Ca 50	0	0
Propyl	3	100	—	—	—	100	—	—	—
Amyl	5	100	Ca 50	0	0	100	Ca 50	0	0
Undecyl	11	100	100	100	0	100	100	100	0
Tridecyl	13	100	100	100	Ca 50	100	100	100	Ca 50

ing the homologs in water with activated carbon and with wheat bunt spores. The data were inconclusive, perhaps because they had no chemical method of measuring the removal. Bioassay, which they used, confounded the results. Let us assume that charcoal or the spores had removed, for example, more of the 17-carbon homolog from the suspension than of the 11-carbon homolog. This would leave a lower dosage of ingredient in the sample of 17-carbon than of 11-carbon homolog. The fungus is about 40 times as sensitive (Wellman and McCallan 29, p. 152, Table I) to the 17- as to the 11-carbon compound. Therefore, the charcoal or the spores would have to remove at least 40 times as much of the 17-carbon compound as of the 11-carbon, if any difference were to show. We suggest that permeation

is the explanation for the difference. If a chemical assay were available, it would probably reveal that spores would remove more of the 17-carbon homolog than of the 11-carbon. Through the courtesy of Dr. Wellman, we have been privileged to study the same series. Data are given in Table IX. Activity increased through 13 carbons. The 17-carbon chain was not tested. The activity of these compounds is being reported in more detail by Rich and Horsfall (24).

McNew and Sundholm (17) published an extensive study of the 4-nitrosopyrazole nucleus. They showed that a hydrocarbon in the 1-position is important. The order of activation was as follows: H < methyl < phenyl- < α-naphthyl.

Through the courtesy of the Naugatuck Chemical Company, we have been privileged to assay a series of 4-nitrosopyrazoles. These are being reported in some detail by Rich and Horsfall (24). Suffice it to say here that 1-phenyl-3,5-dimethyl-4-nitrosopyrazole was much more active than 1,3,5-trimethyl-4-nitrosopyrazole. Also that 3-methyl-5-phenyl-4-nitrosopyrazole was much more active than 3,5-dimethyl-4-nitrosopyrazole. In other words, phenyl is a more active substituent than methyl. One wonders what would be the activity of a cyclohexyl or octyl substituted analog.

DISCUSSION

It is important to remember that the subject of this paper is the fungitoxicity of heterocyclic nitrogen compounds. It is easy to be led away from this by the fact that reactive substituents result in reactive heterocycles. The same reactive groups usually impart activity to many other types of nuclei, however.

At the risk of appearing to overextend a good thing, we would like to suggest that most nitrogen heterocycles are probably *inherently* toxic to fungi. The apparent blandness of some of them, we submit, may well be due to lack of permeation ability. If the compound cannot reach the site of reactivity, it is powerless to react.

A tremendous amount of attention has been devoted to a consideration of the water solubility of fungicides. Goldsworthy and Green (9) tested a series of organic compounds by perfusing the spores with saturated solutions. Wellman and McCallan (29) tried to assay the effect of water solubility of 2-imidazoline compounds by removing them from solution with activated charcoal. Smut spores did not duplicate the results of the charcoal. The agar cup diffusion technique of Oster and Golden (22) assumes the overriding importance of water solubility in fungitoxicity.

Water solubility is, of course, a chemical view of the problem. Presumably, the chemical reactions necessary to inhibit the spore must go on in a water solution; *ergo*, the potency of a good fungicide must be in proportion to its solubility. This conclusion seems to be confirmed by

considering the effect of the polar substituents on toxicity. In general, almost any polar group increases toxicity. But is this all?

If water solubility is the main story, then how explain the action of the aryl and alkyl substituents? Certainly these do not increase water solubility, nor do they provide a reactive group.

A rationale must be devised to account for the simultaneous importance of hydrophobic and hydrophilic groups on the same molecule. We must not forget another assumption that underlies toxicity—that the locus of toxicity is within the cell, that most of the life processes go on inside the cell. If so, then the compound must penetrate through the cell wall and through a semipermeable membrane. Perhaps the addition of a lipophilic substituent enables a potentially toxic molecule to enter a cell where it may realize its potentialities. In this connection the Overton-Meyer theory of drug action must be considered. This theory is based on the assumption that the semipermeable membrane of the cell comprises a fat and water emulsion. The compound penetrates poorly, then, if it is only fat soluble or only water soluble. It should be both. Presumably, that is why most of the successful compounds described herein have a hydrophilic group and a lipophilic group. Only thus can they penetrate the spore rapidly enough to kill it before it can germinate.

Except for 8-quinolinol, not very much is known about the actual mechanism by which heterocyclic nitrogen compounds inhibit spores. It so happens that a new theory of action was proposed by Zentmyer (32, 33) from this laboratory. He postulated that 8-quinolinol inhibited microorganisms by forming chelate salts with the essential metals. This is discussed in detail in the section on the effect of —OH on activity.

Having seen how 8-quinolinol acts to remove by chelation the essential metals from the fungus, one is tempted to extend the idea to other hydroxy-N-heterocyclic compounds. Albert *et al.* (3) did extend the theory to 1-hydroxyacridine and came out with a useful explanation.

It would be interesting then to attempt to make fungitoxic compounds by substituting an hydroxyl group in the 3- or 4-position of pyrazole, 7-position on indole, 1-position on carbazole, or the 1- or 9-position of phenothiazine.

SUMMARY

1. Some 165 heterocyclic nitrogen compounds have been assayed by the spore-germination technique against *Sclerotinia fructicola* and *Macrosporium sarcinaeforme*.

2. Compounds and derivatives of the following types have been investigated: pyrrole, indole, proline, pyrazole, imidazole, thiazole, benzothiazole, pyridine, pyrimidine, hexamethylenetetramine, quinoline, quinine, acridine, phenazine, and phenothiazine.

3. An unsubstituted nucleus containing heterocyclic nitrogen was seldom fungitoxic.

4. The addition of a lipophilic substituent either aryl or alkyl will very often induce toxicity.

5. Toxicity may be produced also by the addition of a polar group such as $—NH_2$, —NO, $—NO_2$, —OH, and —SH.

6. The combination of a hydrocarbon and a polar group sometimes works better than either alone.

7. The suggestion is made that the hydrocarbon substituents probably confer lipoid solubility on the molecule and that the polar groups increase its chemical reactivity. According to the Overton-Meyer theory of toxicity, a really active molecule should have both types of groups.

8. Conversion of the nitrogen in a non-toxic heterocycle to a quaternary ammonium group will almost always impart strong fungitoxicity.

9. A quinoid ring generally has some fungitoxicity. One of the side rings in acridine bears an ortho quinoid position to the heterocycle which may account for the toxicity of acridine over quinoline. If phenothiazine is made *p*-quinoid with oxygen in the 3-position, it becomes toxic. Chlorination did not add fungitoxicity to these nuclei. In some cases chlorination reduced it.

LITERATURE CITED

1. ABIUSSO, N. G. Probable accion fungicida de la fenotiazino y algunos de sus derivados de oxidacion. Rev. Invest. Agric. **2**: 117–138. 1948.
2. ALBERT, A., S. D. RUBBO, R. J. GOLDACRE, and B. G. BALFOUR. The influence of chemical constitution on antibacterial activity. Part III: A study of 8-hydroxyquinoline (oxine) and related compounds. British Jour. Exp. Path. **28**: 69–87. 1947.
3. ALBERT, A., S. D. RUBBO, R. J. GOLDACRE, M. E. DAVEY, and J. D. STONE. The ininfluence of chemical constitution on antibacterial activity. Part II: A general survey of the acridine series. British Jour. Exp. Path. **26**: 160–192. 1945.
4. BARRATT, RAYMOND W., and JAMES G. HORSFALL. Fungicidal action of metallic alkyl bisdithiocarbamates. Connecticut Agric. Exp. Sta. Bull. 508. 51 pp. 1947.
5. CIFERRI, R., e E. E. BALDACCI. Mancata azione antagonista di un sale di zinco sulla fungistaticita dell' 8-idrossichinolina. Il Farmaco **1**: 250–253. 1946.
6. COOLEY, J. S. A study of the physiological relations of *Sclerotinia cinerea* (Bon.) Schröter. Ann. Missouri Bot. Gard. **1**: 291–326. 1914.
7. EVERITT, EDWARD L., and M. X. SULLIVAN. The fungistatic and fungicidal action of certain organic sulfur compounds. Jour. Washington Acad. Sci. **30**: 125–131. 1940.
8. GADDUM, J. H. Reports on biological standards, III. Methods of biological assay depending on a quantal response. Med. Res. Council. London. Spec. Rept. Series **183**: 1–46. 1933.
9. GOLDSWORTHY, M. C., and E. L. GREEN. The fungicidal activity of phenothiazine and some of its oxidation derivatives. Phytopath. **29**: 700–716. 1939.
10. HORSFALL, JAMES G. Fungicides and their action. 239 pp. Chronica Botanica Co., Waltham, Mass. 1945.
11. HORSFALL, J. G., R. A. CHAPMAN, and SAUL RICH. Relation of structure of diphenyl compounds to fungitoxicity. Chem. Biol. Coord. Center of Nat. Res. Council Symposium (in press). 1951.

12. HORSFALL, JAMES G., and SAUL RICH. Oxidized substituents decrease fungitoxicity of reduced compounds. Phytopath. **41**: 18. 1951.
13. HORSFALL, JAMES G., and GEORGE A. ZENTMYER. Fungicidal action of reagents for amino acids, amines, aldehydes, and other reactive cell constituents. Phytopath. **34**: 1004. 1944.
14. HOWARD, F. L., H. L. KEIL, L. WEIL, and C. F. WOODWARD. Nicotinium derivatives as fungicides. Phytopath. **34**: 1004–1005. 1944.
15. LADD, ELBERT C. Parasiticidal preparations. U. S. Patent No. 2,379,723. July 3, 1945.
16. LOCKE, S. B. An isoquinolinium fungicide for apple scab control. Phytopath. **34**. 1008. 1944.
17. MCNEW, GEORGE L., and NORMAN K. SUNDHOLM. The fungicidal activity of substituted pyrazoles and related compounds. Phytopath. **39**: 721–751. 1949.
18. MANTEN, A., H. L. KLÖPPING, and G. J. M. VAN DER KERK. Investigations on organic fungicides. III. The influence of essential trace metals upon the fungitoxicity of tetramethylthiuramdisulfide and 8-hydroxyquinoline. Antonie van Leenwenhoek **17**: 58–68. 1951.
19. MARSH, R. W. Some applications of laboratory biological tests to the evaluation of fungicides. Ann. App. Biol. **25**: 583–604. 1938.
20. MASON, CURTIS L. A study of the fungicidal action of 8-quinolinol and some of its derivatives. Phytopath. **38**: 740–751. 1948.
21. MASON, CURTIS L., and DWIGHT POWELL. A *Pythium* plate method of evaluating fungicides. Phytopath. **37**: 527–528. 1947.
22. OSTER, KURT A., and MILTON J. GOLDEN. Studies on the alcohol-soluble fungistatic and fungicidal compounds. III. Evaluation of the antifungal properties of quinones and quinolines. Jour. Amer. Pharmaceut. Assoc. Sci. Ed. **37**: 429–434. 1948.
23. POWELL, DWIGHT. Copper 8-quinolinolate, a promising fungicide. Phytopath. **36**: 572–573. 1946.
24. RICH, SAUL, and J. G. HORSFALL. Using dosage-response data to distinguish inherent toxicity from permeation. (Delivered at XII Congr. Pure and Applied Chem. 1951.)
25. RIGLER, NEIL E., and GLENN A. GREATHOUSE. Fungicidal potency of quinoline homologs and derivatives against *Phymatotrichum omnivorum*. Indus. & Eng. Chem. **33**: 693–694. 1941.
26. ROBBINS, WILLIAM J., and ILDA MCVEIGH. Effect of hydroxyproline on *Trichophyton mentagrophytes* and other fungi. Amer. Jour. Bot. **33**: 638–647. 1946.
27. STRAIB, W. Über die Wirkung organischer Verbindungen als Spritzmittel gegen Rostpilzinfektion. Zentralb. f. Bakt. Abt. 2. **103**: 73–80. 1941.
28. VICKLUND, R. E., and MILTON MANOWITZ. Mechanism of action of copper 8-quinolinolate. CADO Technical Data Digest **15**: 18–21. 1950.
29. WELLMAN, R. H., and S. E. A. MCCALLAN. Glyoxalidine derivatives as foliage fungicides. I. Laboratory studies. Contrib. Boyce Thompson Inst. **14**: 151–160. 1946.
30. WHIFFEN, ALMA J., NESTOR BOHONOS, and R. L. EMERSON. The production of an antifungal antibiotic by *Streptomyces griseus*. Jour. Bact. **52**: 610–611. 1947.
31. YOUNG, H. C., and C. W. BENNETT. Growth of some parasitic fungi in synthetic culture media. Amer. Jour. Bot. **9**: 459–469. 1922.
32. ZENTMYER, GEORGE A. Mechanism of action of 8-hydroxyquinoline. Phytopath. **33**: 1121. 1943.
33. ———— Inhibition of metal catalysis as a fungistatic mechanism. Science **100**: 294–295. 1944.
34. ZENTMYER, GEORGE A., JAMES G. HORSFALL, and PHILIP P. WALLACE. Dutch elm disease and its chemotherapy. Connecticut Agric. Exp. Sta. Bull. 498. 70 pp. 1946.

PHENOLIC FUNGICIDES IN AGRICULTURE AND INDUSTRY[1]

R. H. Gruenhagen, P. A. Wolf, and E. E. Dunn[2]

Much of Lister's work on the use of phenol as an antiseptic in surgery paralleled Pasteur's memorable development of the germ theory of disease. Lister, although not the first to use an antiseptic, recognized the possible relationship between bacteria and disease. The success he attained in decreasing mortality rates following surgery naturally led to the study of phenol and its derivatives as germicides. These studies are important at the present time even though nearly a century has passed since Lister's work was initiated.

With the growth of information on yeasts, fungi, and viruses as causative agents for many diseases, the study of the effectiveness of phenols against microorganisms has become a continuing subject for investigation. The results of this research now indicate that most phenols have an antimicrobial activity which covers a wide range of organisms. Because of these discoveries it is not surprising that these compounds have become useful in agriculture in combating many plant diseases, as well as in industry for controlling microbial deterioration and spoilage.

Not only do the phenols have wide antimicrobial activity as a class but they also have the advantage of being readily available at favorable prices. Many phenol derivatives are obtained directly as by-products in coal tar distillation and a large group can be synthesized, beginning with benzene as a raw material.

Although there are many derivatives of phenol which are active fungicidally, the scope of this paper will be limited to phenol, halophenols, alkyl and aryl phenols, and esters and salts of phenols.

VERSATILITY OF PHENOLS

Specificity of action rather than breadth of utility has seemed to be the trend of recent developments in the chemical control of microorganisms. With each succeeding new development, the "single shot fungicide" approach to control of microorganisms has receded into the background, and the newer, more specific organic compounds have received the greater attention.

These more specific fungicides and germicides have given better control in limited fields. Although there is much to be said for this approach, there still remains an advantage in being able to work with a single parent

[1] Invitational paper presented before the American Chemical Society in Philadelphia, Pa., on April 12, 1950.

[2] Biochemical Research Department, The Dow Chemical Company, Midland, Michigan.

structure toxic to microorganisms, and to adapt it for use in a number of fields of application against a wide spectrum of microorganisms. Phenol provides a parent structure of this description.

The breadth of effectiveness of three types of phenols, an alkylphenol, a halophenol, and a phenylphenol, is illustrated by the fact that 4-*sec*-butylphenol, 2,4,5-trichlorophenol, and *o*-phenylphenol each inhibit *Escherichia coli* (Mig.) Cast. & Chal., *Salmonella typhosa* (Zopf.) White, *Micrococcus pyogenes* var. *aureus* (Ros'n) Zopf., *Aerobacter aerogenes* (Kruse) Beij., *Bacillus pumilus* Gottheil, *Erwinia carotovora* (Jones) Holl., *Lactobacillus plantarum* (Orla-Jensen) Holl., *Pseudomonas aeruginosa* (Schr.) Mig., *Saccharomyces cerevisiae* Hansen, *Aspergillus niger* v. Tieg., *Chaetomium globosum* Kunze, *Penicillium digitatum* Sacc., *Phomopsis citri* Faw., and *Rhizopus nigricans* Ehr. It is thus seen that each of these chemicals is active against a wide range of bacteria (both gram-negative and gram-positive), and against a number of fungi which are important from an economic standpoint.

ADAPTABILITY OF PHENOLS

The phenols available for a particular application may not, in all cases, give the proper combination of antimicrobial effectiveness, physical

TABLE I

ANTIFUNGAL ACTIVITY OF SEVERAL DERIVATIVES OF 2,4,5-TRICHLOROPHENOL

Derivative	Antifungal activity: Alaska pea emergence, per cent		Inhibition of *Aspergillus niger* 29
	Treated	Control	
Sodium salt	86	71	+
Zinc salt	88	28	No data
Dicyclohexylamine salt	78	45	+
Acetate	85	68	+
Chloroacetate	80	46	+
Benzoate	88	65	+
Parent compound	86	71	+

properties, and physiological properties which are optimum or desirable for that application. In such instances, it is sometimes possible to take a phenol showing the proper microbiological activity and by forming derivatives arrive at a tailor-made molecule which has the proper physical and physiological properties to make it fit more nearly the given usages. The data in Table I show that 2,4,5-trichlorophenol may be converted to various types of salts and esters with different physical properties without seriously impairing its fungicidal properties.

In studies reported earlier from this laboratory, Meuli *et al.* (4) showed that the zinc, lead, copper, potassium, barium, sodium, and mercury salts of 2,4,5-trichlorophenol when formulated as seed protectants all gave a significant degree of disease control on Alaska peas. Although

copper, mercury, and zinc are fungicidal in themselves, their inherent toxicity to microorganisms may in many cases be enhanced when they form a salt with a phenol. Thus the possibilities for combinations having the desired biological and physical properties are greatly increased.

It should not be concluded, however, that all salts and all esters of a given phenol will retain the fungicidal usefulness of the parent compound. Arndt (1) showed that various esters of 2,4,5-trichlorophenol varied in fungicidal activity when tested on cotton seed. He found that in preventing seedling infection of cotton by *Colletotrichum gossypii* South., the acetate of 2,4,5-trichlorophenol was effective, the formate and propionate slightly less effective, and the carbonate, succinate, and laurate were ineffective.

ANTIMICROBIAL ACTIVITY AND CHEMICAL STRUCTURAL RELATIONSHIPS

Although the foregoing observations have emphasized the changes that can be made in phenols with retention of their antimicrobial properties, investigations have shown that in many cases there is an orderly gradation in activity which is related to chemical structural changes. This behavior may often be used to advantage when a high degree of activity for a particular use is desired. An excellent example is the substitution of alkyl or aryl radicals on phenols, altering the activity by the position and type of substituent introduced. Several phenols containing alkyl, cyclohexyl, or phenyl groups as substituents were tested against *Salmonella typhosa*. The results of these tests are summarized in Table II.

TABLE II

BACTERICIDAL ACTIVITY OF SEVERAL ALKYL AND ARYL PHENOLS FOR SALMONELLA TYPHOSA

Substituent on ring	Phenol coefficient for isomers	
	Ortho-substituted	Para-substituted
H	1	1
Methyl	4	5
Ethyl	6	6
Isopropyl	9	10
sec-Butyl	34	51
tert-Butyl	40	33
tert-Amyl	—	12
Cyclohexyl	80	12
Phenyl	26	<1

Although the data presented do not follow smooth progressions, they do indicate significant trends. In general, the bactericidal activity of the series increases as the length of the alkyl chain increases.

A comparison of the ortho-alkylphenol with its corresponding para-alkyl derivative shows that each pair agrees rather well in regard to germicidal activity. Only the phenylphenols and cyclohexylphenols show marked differences between the activity of the corresponding ortho and

para isomers. This relationship between the activity of the corresponding pairs of ortho and para substituted phenols does not hold when the compounds are halogenated. Suter (6) has reviewed the relationships between the structure and germicidal properties of phenols in great detail.

Our knowledge of the effect of position and degree of substitution upon fungistatic effectiveness has been extended by the studies of Marsh and his co-workers (3). They found that bisphenols with halogens in both ortho positions had lower fungicidal activity than those with the halogen in

TABLE III

FUNGISTATIC EFFECTIVENESS OF CHLOROPHENOLS

Phenol	Disease control, per cent emergence		Concentration inhibiting *R. nigricans*, %
	Peanut	Pea	
Unsubstituted	22	63	0.100
2-Chloro-	16	62	0.050
3-Chloro-			0.025
4-Chloro	22	54	0.025
2,3-Dichloro-			0.010
2,4-Dichloro-	28	79	0.010
2,5-Dichloro-			0.010
2,6-Dichloro-			0.025
3,4-Dichloro-			0.010
3,5-Dichloro-			0.005
2,3,4-Trichloro-			0.004
2,4,5-Trichloro-	75	86	0.003
2,4,6-Trichloro-	65	69	0.004
2,3,6-Trichloro-			0.003
2,3,4,6-Tetrachloro-	69	74	0.001
2,3,5,6-Tetrachloro-			0.003
Pentachloro-	67	41	0.0016
Control	24	71	

only one ortho position. They also concluded that too high a halogen content led to low activity.

Our studies of chlorinated phenol showed that biological activity is related to the degree of chlorination.

The chlorinated phenols when used as pea seed protectants against the soil-inhabiting fungi *Pythium*, *Rhizoctonia*, and *Fusarium* gave varying degrees of disease control. Phenol itself and the monochlorophenol isomers, as shown in Table III, caused some injury to pea seed at the dosage used in the tests.

Fungicidal activity was observed, however, with dichlorophenol and increased to a peak with trichlorophenol. As the degree of chlorination increased to tetrachlorophenol the emergence of peas dropped off a little, but fungicidal activity was still present. There was a definite retardation

and injury noted on peas which had been treated with pentachlorophenol.

The trend as a pea seed protectant seems to indicate an increase in fungicidal activity with an increase in chlorination up to tetrachlorophenol, at which point the usefulness in this field decreases. The trend of the chlorophenols as seed protectants on peanuts was similar to that for peas, with the exception of the response to pentachlorophenol. Good disease control and no chemical injury or retardation was noted on peanuts that had been treated with pentachlorophenol.

A series of chlorinated phenols when tested as fungistats against *Rhizopus nigricans* showed a much more uniform progression than when tried against the soil-inhabiting *Pythium*, *Rhizoctonia*, and *Fusarium*. The data in Table III indicate an increase in antimicrobial activity with an increase in the degree of chlorination, leveling off with tetra- and pentachlorophenol.

UTILITY OF PHENOLS

Considering that there is a wide range of phenols with such widespread antimicrobial activity, it is not surprising to find that the phenols have

TABLE IV

ANTIMICROBIAL USES FOR PHENOLS

Application	Phenols used*	Application	Phenols used
Adhesives			
Animal glue	1,2,3,4,5	Oil well drilling mud	2
Starch	2,3,4,6,7		
Vegetable glue	1,2,6,7	Paint	
		Oil base	2,8
Agriculture		Water base	2,3,8 as sodium salts
Bulbs, corms, tubers	1,1b,1c,8		
Dormant fungicides	2,9	Paper and paper products	1,2,2a,3,6,13
Seed protectants	1b,1c		
		Rope and twine	2,6,8
Cooling tower water	2,8		
		Soap, germicidal	14
Disinfectants	3,5,6,7,10		
		Soluble oils	3,8,15
Fruit or vegetable dips	3,6		
		Wood and wood products	
Insulation board	2	Fence posts, poles, ties, etc.	2
Leather processing	2,6,11,12	Interior construction	2,6,8
		Sap stain control	2,6,8

* 1 = 2,4,5-trichlorophenol
1b = zinc salt of 1
1c = acetic acid ester of 1
2 = pentachlorophenol
2a = copper salt of 2
3 = *o*-phenylphenol
4 = chlorocarvacrol
5 = *p*-tertiarybutyl-*m*-cresol
6 = chloro-orthopenylphenol
7 = *p*-chloro-*m*-cresol
8 = 2,3,4,6-tetrachlorophenol
9 = alkanolamine salt of dinitro-ortho-secondary-butylphenol
10 = 2-benzyl-4-chlorophenol
11 = *p*-nitrophenol
12 = *p*-tertiarybutylphenol
13 = 2,4,6-trichlorophenol
14 = 2,2′-methylenebis-(3,4,6-trichlorophenol)
15 = *o*,*m*, and *p*-cresols

utility in a large number of fields. An indication of the breadth of usage is given in Table IV. An examination of the varied uses of phenolic fungicides illustrated in Table IV makes it evident that specific physical properties are often required. Solubility, vapor pressure, stability to heat, stability to light, and resistance to chemical change are properties which determine the persistence of a chemical, that is, its ability to remain at the site of application. Persistence of the proper degree, whether it be high or low, is an important characteristic of an antimicrobial chemical, either in industry or in agriculture.

Physical properties of several phenols are given in Table V. These data illustrate the physical changes produced in phenol by introduction of a phenyl group as a substituent, or by varying the degree of chlorination.

It will be seen from Table V that the variations in physical properties

TABLE V

SOME PHYSICAL PROPERTIES OF PHENOLS

Substituent on nucleus	Melting point, ° C.	Vapor pressure, mm. Hg at 120° C.	Water solubility g./100 g. at 25° C.
None	40.6	96	8.66
o-Phenyl	56.5	2.9	0.070
2,4,5-Trichloro-	67	9.3	0.100
2,3,4,6-Tetrachloro-	69.5	2.7	0.012
Pentachloro-	190	0.8	0.0016

are in orderly relation to the degree of substitution in the phenol nucleus. With increasing substitution the melting point increases, while vapor pressure and solubility decrease. These differences lead to a considerable variation in persistence.

In choosing a fungicide for a field of application, one must consider its physiological effects on plants and animals as well as its physical properties. There are distinctive variations, both qualitative and quantitative, in the physiological properties of the phenols. These variations, along with those differences obtainable in physical properties, give one a versatile set of tools for control of microorganisms.

A consideration of the chlorophenols shows clearly the importance of their individual physical and physiological properties in determining their field of application. Pentachlorophenol is sufficiently persistent to use as a wood preservative under rigorous weathering conditions, while 2,4,5-trichlorophenol is sufficiently fugitive to be an effective, although odorous, fumigant. Pentachlorophenol is sufficiently irritating to the skin to make it necessary to avoid skin contact even with dilute solutions. By contrast, it has been found in experimental trials that 2 per cent solutions of 2,4,5-trichlorophenol can be used safely in the treatment of

athlete's foot. Pentachlorophenol is well known for its effectiveness as a general weed killer; 2,4,5-trichlorophenol is free enough from phytotoxic activity to permit its use as an effective seed fungicide when applied as a 50 per cent dust.

A contrast in physiological properties can be illustrated further by comparing phenol and *o*-phenylphenol; thus, phenol itself is moderate in acute oral toxicity, while *o*-phenylphenol is low in acute oral toxicity. In contact with the skin, phenol is severely irritating, causing burns quite promptly; on the other hand, *o*-phenylphenol causes only minor irritation. Moreover, phenol can be absorbed through the skin rapidly with lethal effects, while *o*-phenylphenol is not absorbed through the skin to an appreciable extent.

Additional studies on rats and dogs have demonstrated that *o*-phenylphenol is quite low in chronic oral toxicity; and tests upon human subjects, using a 5 per cent solution in oil, have shown that *o*-phenylphenol causes neither primary irritation nor sensitization of the skin.

The physiological properties of phenol, combined with its low antimicrobial effectiveness, markedly limit its use. In contrast *o*-phenylphenol has a combination of characteristics such as low toxicity, median persistence, and bactericidal effectiveness over a relatively broad pH range, which make it suitable for use in a wide field of applications, including the food industry. *o*-Phenylphenol is used to control surface contamination of agricultural products and foods when applied in washes or dips, and in wax coatings.

MECHANISM OF ACTION OF PHENOLS

Studies on the mechanisms of fungicidal activity of phenols are limited in number. The report has been made by Zentmyer (9) that 8-hydroxyquinoline is fungistatic because of its property of forming chelate structures with essential trace metal ions. It is hardly likely that this mechanism is responsible for the activity of phenols in general.

The fact that phenols are active against so many bacteria, yeasts, and fungi suggests that these compounds must be interfering with metabolic functions that are essentially common to these organisms. Within the last ten years attention has been focused on the antibiotics and their mode of bactericidal activity. Work in this field has shed light on the general question of the way in which antimicrobial agents act.

The classical work of Woods (8) on the interference of the sulfanilamides with the metabolism of *p*-aminobenzoic acid led to the development of a new research field, the study of metabolic antagonists. From these studies we know that many bactericides function through their ability to interfere with vitamins and other components of essential enzyme systems.

Roberts and Rahn (5) have reported that phenol at lethal concentra-

tions inactivated the energy-producing enzymes present in cultures of *Escherichia coli*. At sublethal concentrations which inhibited growth, the oxidase enzyme system was inactivated. Upon subsequent removal of phenol the oxidase became active again.

Gale and Taylor (2) have shown that phenol, like tyrocidine, causes the loss of internal amino acids from cells of *Streptococcus faecalis* Andrews and Horder. They suggest that the bactericidal properties of these compounds may be explained by their lytic action on the bacterial cell wall. This idea has also been suggested by Tamiya *et al.* (7).

Compounds which interfere with protein metabolism might be expected to have a wide range of antimicrobial activity. The synthesis of protein from inorganic nitrogen or from a limited number of amino acids is a vital function common to yeast, bacteria and fungi. Since there are indications that phenols interfere with protein metabolism, it is not surprising to find that they have a wide spectrum of antimicrobial activity. However, progress toward finding the exact mechanism of action is retarded by inadequate knowledge of protein chemistry.

SUMMARY

Examples have been cited to show that phenols are versatile and adaptable antimicrobial agents. They have a wide antimicrobial spectrum, they can be modified structurally to fit a given usage, and they offer a wide range of physical and physiological properties from which to choose. They are used as microbial inhibitors in a wide number of industrial and agricultural applications.

LITERATURE CITED

1. ARNDT, C. H. An evaluation of certain substituted phenol esters for the treatment of cotton seed. Phytopath. **38**: 978–987. 1948.
2. GALE, E. F., and E. SHIRLEY TAYLOR. Assimilation of amino acids by bacteria. II. Action of tyrocidine and some detergent substances in releasing amino acids from the internal environment of *Streptococcus faecalis*. Jour. Gen. Microbiol. **1**: 77–84. 1947.
3. MARSH, PAUL B., MARY L. BUTLER, and BERNICE S. CLARK. Fungicidal activity of bisphenols. Indus. & Eng. Chem. **41**: 2176–2184. 1949.
4. MEULI, L. J., B. J. THIEGS, and G. E. LYNN. The zinc salt of 2,4,5-trichlorophenol as a seed fungicide. Phytopath. **37**: 474–480. 1947.
5. ROBERTS, MARTHA HOLT, and OTTO RAHN. The amount of enzyme inactivation at bacteriostatic and bactericidal concentration of disinfectants. Jour. Bact. **52**: 639–644. 1946.
6. SUTER, C. M. Relationships between the structures and bactericidal properties of phenols. Chem. Reviews **28**: 269–299. 1941.
7. TAMIYA, HIROSHI, TOMOMICHI YANAGITA, and YOSHIO SUZUKI. New classification of antibacterial substances according to their modes of action. Jour. Penicillin (Japan) **1**: 264–270. 1947. (*Abstr. in* Chem. Abstr. **42**: 5500. 1948).
8. WOODS, D. D. The relation of *p*-aminobenzoic acid to the mechanism of the action of sulfanilamide. Brit. Jour. Exper. Path. **21**: 74–90. 1940.
9. ZENTMYER, GEORGE A. Inhibition of metal catalysis as a fungistatic mechanisim. Science **100**: 294–295. 1944.

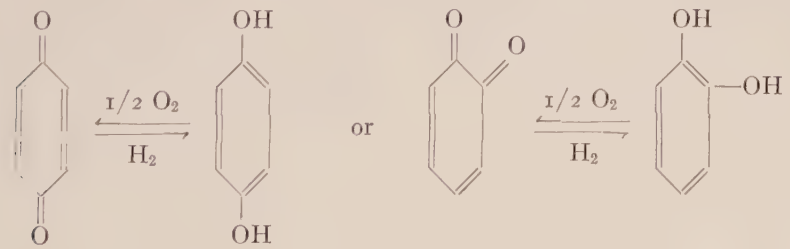
FUNGITOXICITY AND BIOLOGICAL ACTIVITY OF QUINONES[1]

George L. McNew and Harry P. Burchfield

The quinones affect the growth and metabolism of many biological subjects. Although the fungicidal members of this versatile class have found more extensive commercial use, particularly in agriculture, than other representatives, the quinones also affect the growth and metabolism of bacteria, protozoa, higher plants, and mammals. By choice of the appropriate class of quinones and use of suitable substituents it is possible to obtain compounds with desired types of biological activity and physical properties.

The biological activity of quinones may be attributable solely to their strong chemical reactivity but there is reason to believe that they may be particularly adapted to cellular functions. Many quinones have been found naturally in the fungi, bacteria, higher plants, and throughout the animal kingdom so it is entirely possible that they serve some vital function in cell metabolism. If so, the substitution of analogs that would enter into the initial stages of the reaction but not be amenable to its completion would block vital cell processes. On the other hand, the quinones may occur as by-products of some reaction and have very little direct influence on biochemical processes.

Regardless of whether the natural quinones are essential metabolites or mere terminal by-products, they undoubtedly exert an influence on general cell metabolism by their strong oxidation potentials. They, in association with their dihydroxy analogs, constitute a vital redox system that may be important in stabilizing the cell protoplast. Almost any hypothesis for their biological activity would be based on the interconversion of quinones and hydroquinones. The addition of a synthetic



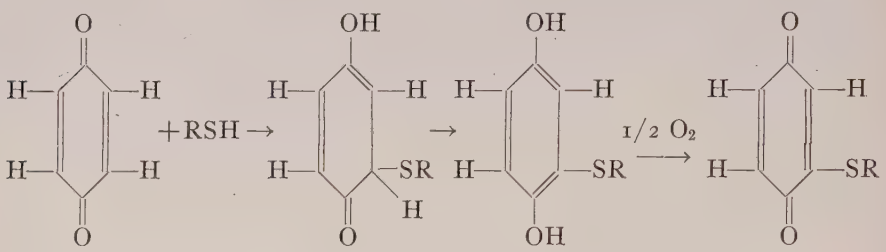
p-benzoquinone hydroquinone *o*-benzoquinone catechol

[1] Invitational paper presented before the American Chemical Society in Philadelphia, Pa., on April 12, 1950.

Copyright, 1951, by Boyce Thompson Institute for Plant Research, Inc.

quinone with strong oxidative ability might shift the oxidation-reduction balance in these systems sufficiently to interfere with normal cell functions.

Another plausible hypothesis is that the biologically active quinones may block vital enzymes. A logical assumption is that enzymes bearing free sulfhydryl groups could form addition products by the following type of reaction:



Sufficient quinones have been tested empirically during the past 15 years to reveal the members that are most active against fungi, bacteria, yeast, malarial parasites, and specific enzyme systems; so it would be in order to draw this information together and see if any consistent correlations exist between biological activity and chemical structure. Data obtained by the writers while at the New York Agricultural Experiment Station, the Naugatuck Chemical Division of U. S. Rubber Co., and Boyce Thompson Institute for Plant Research provide the basis for evaluating the quinones as fungicides. Some of these data have been confirmed and extended by others in numerous field tests.

METHODS OF EXPERIMENTATION AND CLASSIFYING FUNGITOXICANTS

The standard techniques for assaying fungicides were employed with only minor variations. The test tube dilution technique for measuring inhibition of germination in spores of *Alternaria oleracea* Milb. and *Sclerotinia fructicola* (Wint.) Rehm. as recommended by the Committee of the American Phytopathological Society (1) was used as a preliminary screening. Those materials with ED_{50} values in the range .001 to .01 p.p.m. are referred to as AAA compounds, .01 to 0.1 as AA, 0.1 to 1 as A, 1 to 10 as B, 10 to 100 as C, and 100 to 1000 or more as D.

Most compounds were tested for ability to protect tomato foliage from *Alternaria solani* (Ell. & Mart.) J. & G. at 2000 or 1000 p.p.m. Those providing satisfactory control were further tested in dilution series by the method of McCallan and Wellman (50) to determine their effective dosages. Results are expressed as ED_{95} values, determined from plotting the data on logarithmic-probability paper. The experimental error in such tests averaged 15 to 25 per cent.

Field evaluations of fungicides were made by applying materials in

aqueous suspension with sufficient wetting agent (usually Nacconol NR) to permit uniform dispersal. Plants were sprayed at 250 to 400 lb. pressure per square inch until the spray droplets began to coalesce and run on the leaf surface.

THE BENZOQUINONES

Fungicidal activity. Interest in the quinones as fungicides was revived in 1938 when W. P. ter Horst submitted tetrachloro-*p*-benzoquinone and several other materials to J. G. Horsfall and E. Sharvelle of the New York

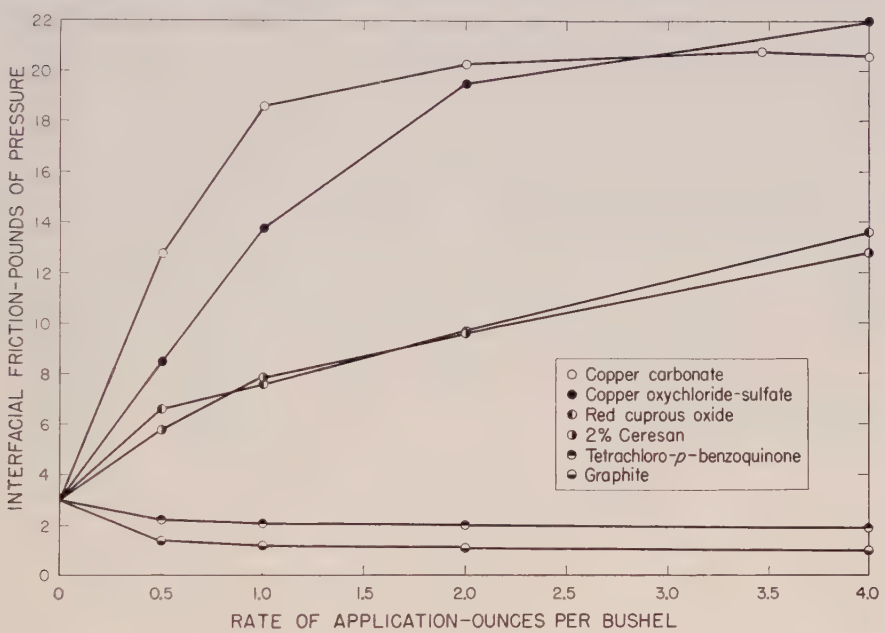


Figure 1. Effect of different seed treatments on interfacial friction of pea seeds.

Agricultural Experiment Station for testing. The material was shown to have many commercial uses as an agricultural fungicide (9, 29, 30, 47, 52, 53, 63, 69, 83). It soon became established under the trade name of Spergon, as the outstanding treatment of legume seed, particularly peas and lima beans. In addition to being effective against most soil-inhabiting fungi and safe for the crops, it did not increase the friction of treated seed and was non-irritating and non-poisonous to handle (51, 54). The material had a lubricating effect somewhat less than graphite (Fig. 1) when tested by the method of Suit and Horsfall (73). This helped facilitate the flow of seed through the cup of a grain drill and eliminated much of the seed injury caused by other treatments as shown in Table I.

The material was not without its disadvantages, however. Before

long it was found to be unsatisfactory as a treatment for spinach and beet seed on some soils, presumably due to its hydrolysis to phytotoxic, water-soluble chloranilic acid and its derivatives. Although excellent results were secured on peat soils, these crops were sometimes injured on alkaline soils of mineral origin.

A wettable grade of tetrachloro-*p*-benzoquinone proved very valuable as a seed bed spray for celery to prevent damping-off of young seedlings before they could become established and to control cabbage downy mildew (16, 25). In the latter capacity, the material restored cabbage plant production to many areas in northern Florida and Georgia where mildew was a limiting factor in plant production. Attempts to use it as a foliage spray for other crops such as tomato, potato, and apple failed because of its poor persistence on foliage, presumably because of its photolability.

TABLE I

EFFECTS OF SEVERAL SEED TREATMENT MATERIALS ON THE RATE OF FLOW THROUGH A FRICTION CUP IN A GRAIN DRILL, SEED COAT INJURY, AND INTERFACIAL FRICTION OF PEA SEEDS (PISUM SATIVUM L. VAR. PERFECTION)

Seed treatment		Interfacial friction, lbs.	Seeds cracked, %	No. of seeds sown per revolution of cup
Material applied	Dosage, oz./bu.			
Copper oxychloride	4	23.5	6.72	97
Copper oxychloride sulfate	4	21.9	6.15	99
Copper carbonate	4	21.2	7.17	97
Cuprous oxide (yellow)	4	19.2	6.19	100
Semesan	4	19.7	4.32	108
Copper formate	4	18.0	4.05	111
Cuprous oxide (red)	4	13.5	3.11	114
2% Ceresan	4	11.9	4.11	107
Cuprious oxide (red)+graphite	4, 2	2.7	0.20	136
Spergon	4	1.9	0.12	142
Graphite	2	1.0	0.02	160
Untreated control	—	3.1	0.15	129

As shown by Wellman and McCallan (80) and confirmed by direct observation, the material does not persist more than seven days when exposed to sunlight and rainfall.

Tetrachloro-*p*-benzoquinone is an AA compound in glass slide tests and has an ED95 of about 400 p.p.m. against *Alternaria solani* on tomato foliage. *p*-Benzoquinone and *p*-toluquinone are only slightly less effective on glass slides but neither controls foliage infection more than 80 per cent at 2000 p.p.m.; so chlorination obviously improved the plant protectant ability of the benzoquinones. The tetramethyl homolog of *p*-benzoquinone (duroquinone) does not show comparable improvement since it is an A compound capable of only 60 per cent control of foliage infection at 2000 p.p.m. Trichloro-*p*-toluquinone is somewhat more effective since it is a B compound with an ED95 of about 1000 p.p.m. on tomato foliage. The 2,5-

and 2,6-dichloro-*p*-benzoquinones are only slightly less effective than tetrachloro-*p*-benzoquinone. Various substituents may be placed in the other two positions of dichloro-*p*-benzoquinone without materially affecting fungicidal activity. In general, the addition of hydroxyl and methoxyl groups resulted in compounds that injure tomato and other plants.

Tetrabromo-*p*-benzoquinone is almost as effective as tetrachloro-*p*-benzoquinone on glass slides but is less effective on tomato foliage. The same relationship holds for the *o*-benzoquinone derivatives whose members are somewhat less effective than their analogs of the *p*-benzoquinone series.

These findings are in general agreement with those of Tappi and Forni (74) except for the fact they concluded that tetrachloro-*o*-benzoquinone was superior to tetrachloro-*p*-benzoquinone. They also noted that substitution of halogen, nitro, alkyl, or alkyloxy groups enhanced the activity of *p*-benzoquinone but reduced activity when substituted on benzoquinone-furoxane. Growth of *Saccharomyces cerevisiae* was 50 per cent inhibited by 10^{-4} to 10^{-5} molar concentrations of benzoquinone derivatives according to Hoffmann-Ostenhof and Fellner-Feldegg (34). 2-Methyl-*p*-benzoquinone, 2-methyl-5-methoxy-*p*-benzoquinone, and 2,6-dimethoxy-*p*-benzoquinone were less active than 2,5-dichloro-*p*-benzoquinone. The quinones may also affect absorption of radioactive phosphate (39).

The naturally-occurring benzoquinones and their reduced analogs. Many substituted benzoquinones and their phenolic analogs have been isolated from fungi and higher plants by Raistrick and others. The subject is adequately reviewed by Mayer (56), Fieser and Fieser (21), and Sexton (68). Erdtman and Rennerfelt (17) have isolated thymoquinone, hydrothymoquinone, carvacrol, and thymol from heartwood of *Tetraclinis articulata*. These materials were decidedly more fungitoxic than phenol; so their presence may explain resistance of these tissues to fungus decay. Fåhraeus (18) believes that wood-rotting fungi regularly oxidize phenols in woody tissue.

The resistance of red onions to smudge was found to be due to the presence of protocatechuic acid by Angell, Walker, and Link (2, 48, 78). A relationship to the quinone analog was not demonstrated. Other naturally-occurring phenolic analogs including hydroquinone and catechol have been associated with disease resistance in plants so detailed studies were made of their fungitoxicity by Greathouse and Rigler (31) who confirmed and extended earlier observations by Wilcoxon and McCallan (82) on the relation of structure to fungicidal activity.

Bacteriostasis by benzoquinones has been studied extensively by several investigators. Oxford (61) found that many benzoquinones related to fumigatin (2-hydroxy-3-methoxy-6-methyl-*p*-benzoquinone from *Aspergillus fumigatus*) and spinulosin were bacteriostatic for *Staphylococcus*

aureus and *Bacterium coli*. The substitution of methoxy groups usually increased activity and their replacement by hydroxyl groups decreased activity. The 3-methoxy, 3,5-dimethoxy, 2,3,5-trimethoxy, 5-hydroxy-3-methoxy, and 1,5-dimethoxy derivatives of 6-methyl-*p*-benzoquinone were decidedly more effective than fumigatin while toluquinone and its hydroxy-substituted derivatives were decidedly less effective.

Page and Robinson (62) were able to correlate the bacteriostatic effects of several *p*-benzoquinone and 1,4-naphthoquinone derivatives for *Staphylococcus aureus*, a gram-positive bacterium, with oxidation potential. A similar relation did not hold for activity against the gram-negative *B. coli;* so, apparently different mechanisms in the two bacteria were affected.

A free position ortho to the carbonyl grouping was found essential to activity against gram-negative bacteria by Geiger (28) who correlated bacteriostasis of a dozen hydroquinone analogs to the stability of the corresponding semiquinone. In general, hydroquinones were found to be more active against gram-positive bacteria. Activity was suppressed by addition of a mercaptan to the quinone. Since one equivalent of sulfhydryl group was required per mole of quinone and only those quinones with unsubstituted positions ortho to the carbonyl were active, the evidence suggests that addition products are formed by the reaction described by Snell and Weissberger (71) and Fieser and Fieser (21).

The bacteriostatic activity of ten benzoquinones was partially blocked by peptone in tests made on *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* by Hoffmann-Ostenhof and Fellner-Feldegg (35). They found many naphthoquinones to be more active than the benzoquinones and the latter fell more or less in order of descending activity: tetrachloro-*p*-benzoquinone, dichlorothymoquinone, 2,6-dimethoxy-*p*-benzoquinone, 3-methoxy-6-methyl-*p*-benzoquinone, thymoquinone, xyloquinone, 2,5-dichloro-*p*-benzoquinone, 2,6-dichloro-*p*-benzoquinone, *p*-toluquinone, and *p*-benzoquinone. These data are in fair agreement with the observations on reaction of fungus spores to most of this series.

The reaction of *Planaria gonocephala* was found to be considerably different from that of bacteria by Hoffmann-Ostenhof and his colleagues (38) who attributed the differences in activity of compounds to different requirements for penetration. Schreier later pointed out (67) that methyl groups reduced activity while methoxyl and hydroxyl groups enhanced activity against this organism.

Effect of benzoquinones on enzyme systems. The halogen-substituted benzoquinones are more effective than other members of this group in inhibiting carboxylase. In descending order of effectiveness according to Kuhn and Beinert (45) the compounds were: tetrachloro-*p*-benzoquinone, *p*-benzoquinone, *p*-toluquinone, acetamino-*p*-benzoquinone, 2,5-dimethyl-

p-benzoquinone, 2,6-dimethoxy-*p*-benzoquinone, tetramethyl-*p*-benzoquinone (duroquinone), fumigatin, tetrahydroxy-*p*-benzoquinone, and 2,5-dihydroxy-3,6-dinitrobenzoquinone. Inhibition at 3.7×10^{-6} ranged from 94 to 0 per cent. Hoffmann-Ostenhof and Kriz (41) found about 15 per cent inhibition of glucose fermentation at about the same concentration of thymoquinone and toluquinone. They also found tetrachloro-*p*-benzoquinone to be the most active benzoquinone for preventing decarboxylation of pyruvic acid. It was followed by 3-methoxy-6-methyl-*p*-benzoquinone, dichloro-*p*-benzoquinone, and *p*-benzoquinone.

Some benzoquinones inactivate serum cholinesterase and pancreatic lipase but others do not. The failure of these sulfhydryl bearing enzymes to react with biologically active quinones should be investigated more thoroughly according to Hoffmann-Ostenhof *et al.* (37) who point out that sulfhydryl groups may enter into any one of three types of reactions.

The lipide formation in yeast (40) may be affected by benzoquinone derivatives. A concentration of 10^{-4} molar of toluquinone or 3-methoxy-6-methyl-*p*-benzoquinone stimulates production. Other enzymes affected by benzoquinone derivatives are catalase (36), proteinase (43), and phosphatase (64). In addition to these effects on enzymes, the quinones may alter the permeability of yeast and beet cells (39, 42).

THE NAPHTHOQUINONES

As a general rule the naphthoquinone compounds are more active than benzoquinone derivatives as fungitoxicants, bacteriostats, and enzyme inhibitors. Shortly after tetrachloro-*p*-benzoquinone was introduced as an agricultural fungicide, ter Horst and Felix (75) announced that 2,3-dichloro-1,4-naphthoquinone was about four to eight times as effective. This material, under the trade name of Phygon (formerly Compound 604), proved to be an effective seed protectant. In addition it provided excellent protection to foliage and fruit when used at concentrations of 1 lb. or less per 100 gallons of spray mixture. It persisted very well since it was not so photolabile as tetrachloro-*p*-benzoquinone. The efficiency of the material is very dependent upon particle size since optimum protection of surface areas is obtained from particles of 3 to 5μ radius (5, 6, 55). The principal difficulties encountered in exploiting the exceptional properties of this material (8, 14, 15, 30, 46, 47, 59, 63, 70) have arisen from its tendency to be phytotoxic to some crops in some areas and its tendency to cause a dermatitis resembling sunburn on some sensitive individuals.

2,3-Dichloro-1,4-naphthoquinone is one of the more active organic fungicides described to date since it classifies as AAA to AA compound on glass slides and has an ED95 value of 80 to 100 p.p.m. against *Alternaria solani* on tomato foliage. It is a very versatile fungicide controlling such fungi as *Pythium* spp. (seed-decay and damping-off of vegetables),

Phytophthora infestans (late blight of potatoes), *Venturia inaequalis* (apple scab), *Sclerotinia fructicola* (brown rot of stone fruits), *Colletotrichum lindemuthianum* (bean anthracnose), and *Gymnosporangium juniperi-virginianae* (cedar-apple rust), and many others.

Some of the same general relations between chemical structure and fungitoxicity hold in the naphthoquinones as in the benzoquinone series. Halogenation improves their effectiveness. For example, 1,4-naphthoquinone is an A compound on glass slides with an ED95 on tomato foliage of about 1000 p.p.m. 2-Chloro-1,4-naphthoquinone is an AA compound with an ED95 of over 1000 p.p.m. and 2,3-dichloro-1,4-naphthoquinone is an AAA compound with an ED95 of about 100 p.p.m. on tomato foliage. The 1,2-naphthoquinone derivatives are about equally fungitoxic since the parent material is an A to AA compound with an ED95 of about 800 p.p.m. and the 3-chloro derivative is an AA compound with an ED95 by more than 2000 p.p.m.

The naturally-occurring naphthoquinones are fungitoxic. 2-Methyl-1,4-naphthoquinone, a compound with vitamin K activity, is an A to AA compound with an ED95 of about 2000 p.p.m. Juglone (5-hydroxy-1,4-naphthoquinone) which occurs naturally in walnut shell and bark along with the corresponding trihydroxynaphthalene is a potent fungicide as reported by Gries (32). It is an AA compound on glass slides and has an ED95 value of 1000 p.p.m. Phthiocol (2-methyl-3-hydroxy-1,4-naphthoquinone), a constituent of the tubercle bacillus, is an A compound with an ED95 greater than 2000 p.p.m.

Little, Sproston, and Foote (49) found that 2-methoxy-1,4-naphthoquinone, which they isolated from *Impatiens balsamina*, was highly fungitoxic. Several other substituted 1,4-naphthoquinone compounds were more active. The descending order of effectiveness was found to be: 2,3-dichloro-, 2-amino-, 2-mercapto-, unsubstituted, 2-methoxy-, and 2-amino- derivatives of 1,4-naphthoquinone. Fungitoxicity was so closely correlated with ability to inhibit carboxylase that they suggested (24) use of this test for screening purposes. Molho and Lacroix (57) noted that 2-methoxy-naphthoquinone did not affect growth of *Aspergillus niger* in Rawlins medium but that inhibitors such as 2-methyl-1,4-naphthoquinone and 2-chloro-1,4-naphthoquinone antagonized each other sufficiently to prevent most of the inhibition.

Solanione, the purple pigment, from *Fusarium solani*, interferes with fat formation in closely related, non-pigmented fungi such as *F. lini* (60). The substitution of a methyl or hydroxyl group into 1,4-naphthoquinone increased the toxic effects of the compounds; so Weiss, Fiore, and Nord (79) studied the effect of several naphthoquinones on the growth and lipide production from carbohydrates in liquid culture. There was no correlation between fungitoxicity and redox potential. The ED50 for

Saccharomyces cerevisiae in glucose peptone broth ranged from about 10^{-6} molar for methyl naphthazarin through 2,3-dichloro-1,4-naphthoquinone, phthiocol, naphthazarin, 2-chloro-1,4-naphthoquinone, 1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone to 10^{-4} molar for 2-methyl-1,4-naphthoquinone and 1,2-naphthoquinone in tests made by Hoffmann-Ostenhof and Fellner-Feldegg (34).

Effect on bacteria. The naphthoquinones affect the growth and survival of bacteria in pure cultures. 2-Methyl-1,4-naphthoquinone (a compound having vitamin K activity) will destroy both gram-negative and gram-positive bacteria (7) but apparently has a wider spectrum of activity in liquid media than on solidified media according to Mulé (58). The activity of this compound is attributed to its ability to add sulfhydryl groups in the 3-position by Colwell and McCall (7) who noticed that equimolar concentrations of thioglycolic acid, ethyl mercaptan, or cysteine hydrochloride neutralized the bacteriostatic activity. The hydroquinone analog and other naphthoquinone derivatives are also effective against many bacteria (Del Vecchio *et al.*, 11, 12).

Unsubstituted 1,2-naphthoquinone was more inhibitory than 1,4-naphthoquinone for *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* on different media in tests made by Hoffmann-Ostenhof and Fellner-Feldegg (35). They also found that chlorination increased the activity so that 2,3-dichloro-1,4-naphthoquinone, 2-chloro-1,4-naphthoquinone, and 2-chloro-3-hydroxy-1,4-naphthoquinone were the more active compounds tested while phthiocol, 2-methyl-1,4-naphthoquinone, and methyl naphthazarin were mediocre and much less effective than they were against yeast. Various naphthoquinones affect respiration and light emission by *Photobacterium phosphoreum* according to Spruit and Schuiling (72).

Although there is only minor variation between the reactions of bacteria and fungi to naphthoquinone compounds, it might be pointed out that the effects on photosynthesis in algae (10, 26, 27, 66) are entirely different. The unsubstituted 1,4-naphthoquinone is most inhibitory (66), followed in decreasing order by the 2-chloro-, 2-ethyl-, 2-methyl-, 2,3-dimethyl-, and 5-hydroxyl- derivatives.

Effect on animal life. Some of the outstanding research on the relationship of chemical structure to biological activity in the naphthoquinone series was done on malarial parasites by Fieser and his coworkers. The more active members against *Plasmodium* were derivatives of 2-hydroxy-1,4-naphthoquinone with alkyl substituents in the 3-position. The length of side chain influences the potency of the nucleus (22) apparently by affecting solubility.

The number of carbon atoms in the side chain required for maximum activity varied from 9 for an alkyl group to 15 with a phenylalkyl group.

The relationships were found to be directly correlated with solubility distribution characteristics between ether and aqueous buffers. The more active members in each series had a pE of 10 to 12, which is the pH of a buffer that extracts 1/101 part of quinone from an equal volume of ether solution (19).

Some of the compounds that were active in ducks, apparently by preventing respiration, were relatively inactive in humans. The plasma of human beings was found to be much more capable of binding naphthoquinones than that of monkeys, ducks, or chicks (33). The alkyl side chains in the 3-position were found by Fieser *et al.* (20) to be oxidized to secondary or tertiary alcohols or carboxylic acids. The first point of attack was in the terminal point of the chain. Some of the oxidation products derived from feeding human subjects 0.5 to 4.0 g. daily for two weeks were about one-tenth as active as the unoxidized starting material.

Bueding, Peters, and Waite (4) also found proteins to be capable of inhibiting derivatives of 1,4-naphthoquinone during the course of studies on the effect of 2-methyl-1,4-naphthoquinone on glycolysis of *Schistosoma*. These compounds also affect the velocity and amplitude of nerve reaction to electric shock (Torda and Wolff, 77), apparently because of its inhibition of choline acetylase at concentrations greater than 10^{-5} molar (76). A heavier concentration is required to affect other enzymes or to interfere with acetyl choline. Some naphthoquinones may be degradated to nonquinoid substances by oxidation with enzymes of dog liver (23).

Effect on enzyme systems. In addition to the enzymic degradation and effects on cholinesterases mentioned above, the naphthoquinones may interact with many enzymes. Hoffmann-Ostenhof and Moser (43) found the naphthoquinones were more active than benzoquinones in preventing liquefaction of gelatine by yeast proteinase.

Detailed studies on carboxylase inhibition by Kuhn and Beinert (45) showed that derivatives of 1,4- and 1,2-naphthoquinone were equally active. Substitution of a chlorine or bromine atom in the 2-position increased the activity of both naphthoquinones. Methyl or ethyl substitution in the 2-position of 1,4-naphthoquinone suppressed activity somewhat while hydroxyl and methoxyl groups were even more serious. Phthiocol and other naphthoquinones with methyl groups in the 3-position and hydroxyl groups in the 2-position were relatively inactive at a concentration of 1.5×10^{-5} molar. Hydroxyl groups substituted in the 5- and 8-positions as in juglone and naphthazarin did not suppress activity since these materials were more active than naphthoquinone. The 2-methyl and 2-hydroxyl derivatives of 5,8-dihydroxy-1,4-naphthoquinone were even more active. It was estimated that only seven mols of 2-bromonaphthazarin, the most active member of the series, were required to inactivate a mol of carboxylase.

The naphthoquinones affect respiration of various types of cells. Respiration of algae may be accelerated by 2-methyl-1,4-naphthoquinone at concentrations that are inhibitory to photosynthesis (26). Gaffron points out that vitamin K may be present to the extent of 10^{-3} molar in plants and undoubtedly serves as a catalyst and regulator of cell functions such as the adaptation to anaerobic utilization of hydrogen by *Scenedesmus*.

Wendel (81) found that the decrease in oxygen uptake caused by 2-hydroxy-3-alkyl-naphthoquinones was due to suppression of carbohydrate oxidation. Lactic acid accumulates so interference is experienced in conversion of lactates to pyruvate. Of 76 compounds tested he found 69 inhibited respiration at concentrations roughly paralleling their antiplasmodial activity. The seven compounds that did not correlate well had aryl groups substituted on the alkyl side chain.

Oxygen uptake by the succinate oxidase system is blocked by 3×10^{-6} molar concentration of these compounds according to Ball *et al.* (3). Cytochrome oxidase, catalase, pyridine nucleotide systems, and urease were unaffected. They concluded that these materials interfere with oxidation of reduced cytochrome *b* but not with the reduction process. Fieser (19) believes that this interference with respiratory processes by the 2-hydroxy-3-alkyl-naphthoquinones is the mechanism of their antimalarial activity even though there is no correlation between their redox potential and antiplasmodial activity. There is some sort of union with protein constituents but this is not through a primary valence since the quinone may be released by alcohol but not by dialysis.

The lipase activity of molds is affected by naphthoquinones such as solanione. Fat production by *Fusarium lycopersici* and *F. solani* was increased by nutritional conditions that changed the intensity of pigmentation in experiments conducted by Nord, Fiore, and Weiss (60). Solanione does not adversely affect pigmented species of Fusaria such as *F. lycopersici* but Deschamps (13) found that it increased the desaturation of fats in non-pigmented species presumably by affecting the hydrogen transport system.

Neither 1,2- nor 1,4-naphthoquinone affected the total lipide content of yeast in experiments of Hoffmann-Ostenhof and Kriz (40), but naphthazarin stimulated production. The proportion of sterols was increased.

Other enzymes may be affected by naphthoquinone and its derivatives. Among these are phosphomonoesterase (44), urease (65), and enzymes regulating aerobic glycolysis (4).

OTHER QUINONES

The anthraquinones have not proved to be good fungicides. Neither have the higher members of the quinone series with more than three rings. Apparently very few biologically active members have been found

among these higher members of the series since they are mentioned infrequently in the literature (68).

9,10-Phenanthraquinone, however, is a potent fungicide. It is an AA compound on glass slide spore germination tests and has an ED95 of about 400 p.p.m. in foliage protection tests with *Alternaria solani*. Effective control of apple scab and other foliage diseases can be secured by application of sprays containing 3 to 4 lb. per 100 gallons of water. The high dosage rate required for disease control has discouraged its commercial use. Retenequinone is relatively non-toxic to fungus spores on glass slides or tomato foliage.

DISCUSSION AND SUMMARY

Sufficient data are available to demonstrate that the quinones are among the more potent and versatile groups of biological chemicals if the proper substituents are present in the molecule. Although there are exceptions to any broad rules that may be formulated, the following generalizations are usually true.

1. Those materials that are fungitoxic to plant parasites usually exert an adverse effect on yeasts and bacteria.

2. The various quinone nuclei vary in potency from anthraquinone $<$benzoquinone$<$phenanthraquinone$<$naphthoquinone but the substituents on the nucleus affect their activity so much that the classes overlap appreciably.

3. Halogenation generally improves the fungitoxicity, decreases phytotoxicity, and renders the materials less soluble so the halogenated members are better qualified for use in agriculture than other members. Fungitoxicity and bacteriostasis are usually correlated with extent of halogenation and as a general rule Cl$>$Br$>$I in any series of analogs.

4. Hydroxyl and methoxyl groups ortho to the carbonyl groups usually increase solubility and phytotoxicity to higher plants.

5. The substitution of an alkyl group ortho to the carbonyl group usually reduces fungitoxicity.

6. The combination of two alkyl groups, an alkyl group and a hydroxyl or alkyloxy group between two carbonyl groups para to each other, destroys most of the activity for fungi, bacteria and enzyme systems. Such combinations where separated as in the 3,5-positions of a benzoquinone or removed from position ortho to the carbonyl groups as in 5,8-dihydroxy-1,4-naphthoquinone or its 2-methyl derivative do not destroy activity.

7. In general, *p*-quinone compounds are only moderately, if at all, more active than their ortho analogs.

The reasons for these relationships have not been fully explored. Not enough is known about the solubilizing effects of different substituents and the character of cell walls to be penetrated to separate the basic infor-

mation into factors affecting penetration and those affecting vital cell functions except for the detailed studies on antimalarial naphthoquinones by Fieser (19) and his coworkers.

Although the quinones are primarily characterized by strong oxidation potentials, oxidation alone will not explain the effects obtained. *p*-Benzoquinone with a potential of .7 is not more fungitoxic than 1,4-naphthoquinone, or 9,10-phenanthraquinone with E_0 values of .46 but all three are more active than 9,10-anthraquinone with E_0 of .154. There is fair correlation between bacteriostasis and oxidation potential in some gram-positive bacteria but not in gram-negative bacteria, *Plasmodium*, or fungi.

One cannot deny the potential stabilizing value of the quinone-hydroquinone system in living cells, particularly when they are found so commonly in fungi, higher plants, bacteria, and other entities. There is every reason to believe that the quinones may serve as part of the terminal oxidase system for example in the oxidation (68) of phenylalanine, tyrosine, and other cell constituents. Any disturbance in the cell balance by introduction of a highly oxidized compound would upset the normal balance of the cell.

Since no one has attributed a specific role other than vitamin K activity to the quinones in cellular metabolism, there is no good basis for postulating that the introduced quinones serve as antimetabolites. There is fair evidence in the studies by Woolley (84) and Hoffmann-Ostenhof and Fellner-Feldegg (34) that certain halogenated quinones may compete with 2-methyl-naphthoquinone in cell functions. Further research along this line may reveal a vital function for quinones as was done for *p*-aminobenzoic acid after it was found to be antagonized by sulfanilamide drugs.

The importance of the addition reaction between quinones and sulfhydryl-bearing enzymes has not been fully established because the terminal products have not been identified. The reaction, undoubtedly, is of vital importance because it directly affects both vital syntheses and respiration. It has been fully established that such reactions do occur (71), the activity of quinones is suppressed by equimolar addition of thiol compounds (7, 28), and quinones containing alkyl mercapto groups ortho to the carbonyl group are relatively less active than their unsubstituted homologs. Further evidence in support of the hypothesis may be derived from the fairly common observation that the substitution of an alkyl, or similar stable group, ortho to one of the carbonyl groups reduces activity while substitution of two such groups intermediate between para-carbonyl groups practically eliminates fungitoxicity and bacteriostasis.

Simple addition reactions, however, will not explain all the data available. The halogenated quinones are more active against microorganisms and react more readily with enzymes than unsubstituted members; so

the hypothesis would have to be extended to include substitution reactions. Another and more serious discrepancy in the hypothesis is that some sulfhydryl-bearing enzymes are not inactivated by quinones which should readily form addition products. This may be due to a strong oxidative reaction on sulfhydryl groups to produce disulfide linkages as intimated by Hoffmann-Ostenhof *et al.* (37).

There is enough evidence available to indicate that more than one—possibly three—mechanisms of action are involved in the effects of quinones: binding of enzymes to the quinone nucleus by substitution or addition at the double bond, an oxidative effect on sulfhydryl enzymes, or a change in redox potential in some subjects such as the gram-positive bacteria.

LITERATURE CITED

1. AMERICAN PHYTOPATHOLOGICAL SOCIETY. COMMITTEE ON STANDARDIZATION OF FUNGICIDAL TESTS. Test tube dilution technique for use with the slide-germination method of evaluating protectant fungicides. Phytopath. **37**: 354–356. 1947.
2. ANGELL, H. R., J. C. WALKER, and KARL PAUL LINK. The relation of protocatechuic acid to disease resistance in the onion. Phytopath. **20**: 431–438. 1930.
3. BALL, ERIC G., CHRISTIAN B. AUFINSEN, and OCTAVIA COOPER. The inhibitory action of naphthoquinones on respiratory processes. Jour. Biol. Chem. **168**: 257–270. 1947.
4. BUEDING, ERNEST, LAWRENCE PETERS, and JEAN F. WAITE. Effect of 2-methyl-1,4-naphthoquinone on glycolysis of *Schistosoma mansoni*. Proc. Soc. Exp. Biol. & Med. **64**: 111–113. 1947.
5. BURCHFIELD, H. P., DELORA K. GULLSTROM, and G. L. MCNEW. Agricultural dusts. Preparation of dusts of uniform particle size by fractional sedimentation. Analyt. Chem. **20**: 1168–1173. 1948.
6. BURCHFIELD, H. P., and GEORGE L. MCNEW. Mechanism of particle size effects of fungicides on plant protection. Contrib. Boyce Thompson Inst. **16**: 131–161. 1950.
7. COLWELL, CHARLOTTE A., and MARY MCCALL. Studies on the mechanism of antibacterial action of 2-methyl-1,4-naphthoquinone. Science **101**: 592–594. 1945.
8. CRALLEY, E. M. Rice seed treatments. Arkansas Agric. Exp. Sta. Rept. Ser. No. 5. 1947.
9. CUNNINGHAM, H. S., and E. G. SHARVELLE. Organic seed protectants for lima beans. Phytopath. **30**: 4–5. 1940.
10. DAM, HENRIK. Vitamin K in unicellular photosynthesizing organisms. Amer. Jour. Bot. **31**: 492–493. 1944.
11. DEL VECCHIO, G., V. DEL VECCHIO, e R. ARGENZIANO. Sull'azione antibiotica del chinoni. V. L'attività antibiotica in vitro del 2-metil-1,4-naftoidrochinone nei confronti di diverse specie microbiche. Boll. Soc. Ital. Biol. Sper. **24**: 190–191. 1948.
12. DEL VECCHIO, G., V. DEL VECCHIO, C. CARRATÙ, A. NAPOLI, e R. ARGENZIANO. Sull'azione antibiotica del chinoni. VIII. Ulteriori ricerche sull'attività antibiotica in vitro del 2-metil-1,4-naftochinone verso le *Brucelle*. Boll. Soc. Ital. Biol. Sper. **24**: 1167–1168. 1948.
13. DESCHAMPS, IGNACIO. On the mechanism of enzyme reaction. XXXIV. The influence of a pigment from *Fusarium solani* D_2 purple (solanione) on the composition of fats formed in Fusaria. Arch. Biochem. **20**: 457–464. 1949.
14. DUNEGAN, JOHN C., and M. C. GOLDSWORTHY. The control of blossom blight and its relation to brown rot of Red Bird peaches at harvest. Plant Disease Reptr. **32**: 136–137. 1948.

15. Dunegan, John C., John W. Roberts, and M. C. Goldsworthy. Spray experiments with organic fungicides for the control of apple bitter rot. Plant Disease Reptr. **28**: 1035–1037. 1944.
16. Eddins, A. H. Control downy mildew of cabbage with Spergon. Florida Agric. Exp. Sta. Press Bull. 633. 4 pp. 1947.
17. Erdtman, Holger, and Erik Rennerfelt. Fungicidal properties of some constituents of the heartwood of *Tetraclinis articulata*. Acta Chem. Scand. **3**: 906–911. 1949. (*Abstr. in* Chem. Abstr. **44**: 3651. 1950).
18. Fåhraeus, Gösta. The oxidation of phenolic compounds by wood-rotting fungi. Kgl. Lantbruks Högskol. Ann. **16**: 618–629. 1949. (*Abstr. in* Chem. Abstr. **44**: 707. 1950.)
19. Fieser, Louis F. Naphthoquinones as antimalarial and inhibitors of respiration. Trans. 3rd Conf. Biol. Antioxidants. p. 24–29. 1948.
20. Fieser, L. F., F. C. Chang, W. C. Dauben, C. Heidelberger, H. Heymann, and A. M. Seligman. Naphthoquinone antimalarials. XVIII. Metabolic oxidation products. Jour. Pharm. & Exp. Therapeutics **94**: 85–96. 1948.
21. Fieser, Louis F., and Mary Fieser. Organic chemistry. 1091 pp. D. C. Heath & Co., Boston. 1944.
22. Fieser, Louis F., and Hans Heymann. Naphthoquinone antimalarials. XXII. Relative antirespiratory activities (*Plasmodium lophurae*). Jour. Biol. Chem. **176**: 1363–1370. 1948.
23. Fieser, L. F., H. Heymann, and A. M. Seligman. Naphthoquinone antimalarials. XX. Metabolic degradation. Jour. Pharm. & Exp. Therapeutics **94**: 112–124. 1948.
24. Foote, Murray W., John E. Little, and Thomas J. Sproston. On naphthoquinones as inhibitors of spore germination of fungi. Jour. Biol. Chem. **181**: 481–487. 1949.
25. Foster, H. H. Control of downy mildew or "rust" in cabbage seedbeds. Mississippi Agric. Exp. Sta. Circ. 127. 7 pp. 1946.
26. Gaffron, H. Some effects of derivatives of vitamin K or the metabolism of unicellular algae. Jour. Gen Physiol. **28**: 259–268. 1945.
27. ———— *o*-Phenanthroline and derivatives of vitamin K as stabilizers of photoreduction in *Scenedesmus*. Jour. Gen. Physiol. **28**: 269–285. 1945.
28. Geiger, Walton B. The mechanism of antibacterial action of quinones and hydroquinones. Arch. Biochem. **11**: 23–32. 1946.
29. Gould, C. J. Vegetable seed-treatment tests in western Washington, 1943. Plant Disease Reptr. **27**: 594–601. 1943.
30. ———— Our experience with seed treatments in western Washington agriculture. Western Canner & Packer **37**: 47–51. April 1945.
31. Greathouse, Glenn H., and Neil E. Rigler. The chemistry of resistance of plants to *Phymatotrichum* root rot. IV. Toxicity of phenolic and related compounds. Amer. Jour. Bot. **27**: 99–108. 1940.
32. Gries, Geo. A. Juglone (5-hydroxy-1,4-naphthoquinone)—A promising fungicide. Phytopath. **33**: 1112. 1943.
33. Heymann, Hans, and Louis F. Fieser. Naphthoquinone antimalarials. XIX. Antirespiratory study of protein binding. Jour. Pharmacol. & Exp. Therapeutics **94**: 97–111. 1948.
34. Hoffmann-Ostenhof, O., und H. Fellner-Feldegg. Die Hemmung des Hefewachstums durch verschiedene Chinone. XIV. Mitteilung über bakteriostatische Chinone und andere Antibiotica. Akad. Wiss. Wien Math.-Naturwiss. Kl. Sitzungsber. Abt. IIb. **158**(7/8): 648–654. 1949.
35. ———— Die Hemmung des Wachstums verschiedener Bakterienarten durch Chinone. XVI. Mitteilung über bakteriostatische Chinone und andere Antibiotica. Akad. Wiss. Wien Math.-Naturwiss. Kl. Sitzungsber. Abt. IIb.**158**(7/8): 720–729. 1949.

36. HOFFMANN-OSTENHOF, O., und S. GIERER. Untersuchungen über bakteriostatische Chinone und andere Antibiotica. IX. Mitteilung: Hemmung der Aktivität kristallisierter Katalase aus Rinderleben durch Chinone und Indophenole. Akad. Wiss. Wien Math.-Naturwiss. Kl. Sitzungsber. Abt. IIb. **157**(9): 379–384. 1948.

37. HOFFMANN-OSTENHOF, O., ELISABETH BIACH, S. GIERER, und O. KRAUPP. Untersuchungen über bakteriostatische Chinone und andere Antibiotica. XIII. Mitteilung: Durch Chinone nicht beeinflussbare Esterasen. Akad. Wiss. Wien Math.-Naturwiss. Kl. Sitzungsber. Abt. IIb. **157**(10): 576–580. 1948.

38. HOFFMANN-OSTENHOF, O., L. BERTALANAFFY, und O. SCHREIER. Untersuchungen über bakteriostatische Chinone und andere Antibiotica. VII. Mitteilung: Quantitative Versuche über die Giftwirkung von Chinonen auf *Planaria gonocephala*, einen Strudelwurm aus der Ordnung der *Tricladida*. Akad. Wiss. Wien Math.-Naturwiss. Kl. Sitzungsber. Abt. IIb. **157**(5): 61–71. 1948.

39. HOFFMANN-OSTENHOF, O., und ELSE KRIZ. Untersuchungen über bakteriostatische Chinone und andere Antibiotica. X. Mitteilung: Studien über das Verhalten pflanzlicher Zellen gegenüber Chinonen. Akad. Wiss. Wien Math.-Naturwiss. Kl. Sitzungsber. Abt. IIb. **157**(9): 410–420. 1948.

40. ———— The influence of some quinone derivatives on the lipide formation in yeast. Arch. Biochem. **24**: 459–461. 1949.

41. ———— Der Einfluss von Chinonen auf verschiedene Stoffwechselfunktionen der Hefe. XV. Mitteilung über bakteriostatische Chinone und andere Antibiotica. Akad. Wiss. Wien Math.-Naturwiss. Kl. Sitzungsber. Abt. IIb. **158**(7/8): 678–693. 1949.

42. ———— Der Einfluss von Chinonen und anderen Wirkstoffen auf die Resorption von radioaktiven Phosphat durch Hefe. XVII. Mitteilung über antibakterielle Chinone und andere Antibiotica. Akad. Wiss. Wien Math.-Naturwiss. Kl. Sitzungsber. Abt. IIb. **159**(1/2): 90–100. 1950.

43. HOFFMANN-OSTENHOF, und H. MOSER. Untersuchungen über bakteriostatische Chinone und andere Antibiotica. XII. Mitteilung: Hemmungswirkungen verschiedener Chinone auf die Eiweissspaltung durch Hefeproteinasen. Akad. Wiss. Wien Math.-Naturwiss. Kl. Sitzungsber. Abt. IIb. **157**(10): 570–575. 1948.

44. HOFFMANN-OSTENHOF, O., und ELISABETH PUTZ. Untersuchungen über bakteriostatische Chinone und andere Antibiotica. XI. Mitteilung: Hemmwirkungen verschiedener Chinone auf einige Phosphomonoesterasen der Hefe. Akad. Wiss. Wien Math.-Naturwiss. Kl. Sitzungsber. Abt. IIb. **157**(9): 421–425. 1948.

45. KUHN, RICHARD, und HELMUT BEINERT. Hemmstoffe der Carboxylase. Chem. Ber. **80**: 101–109. 1947.

46. LEACH, L. D. Seed-borne *Phoma* and its relation to the origin of sugar beet seed lots. Proc. Fourth Gen. Meet. Amer. Soc. Sugar Beet Tech. **1946**: 381–388. 1947.

47. LEUKEL, R. W., and J. E. LIVINGSTON. Smut control in sorghum and effect of dust fungicides and storage on emergence. Phytopath. **35**: 645–653. 1945.

48. LINK, KARL PAUL, H. R. ANGELL, and J. C. WALKER. The isolation of protocatechuic acid from pigmented onion scales and its significance in relation to disease resistance in onions. Jour. Biol. Chem. **81**: 369–375. 1929.

49. LITTLE, JOHN E., THOMAS J. SPROSTON, and MURRAY W. FOOTE. Isolation and antifungal action of naturally occurring 2-methoxy-1,4-naphthoquinone. Jour. Biol. Chem. **174**: 335–342. 1948.

50. MCCALLAN, S. E. A., and R. H. WELLMAN. A greenhouse method of evaluating fungicides by means of tomato foliage diseases. Contrib. Boyce Thompson Inst. **13**: 93–134. 1943.

51. MCGAVACK, T. H., L. J. BOYD, R. TERRANOVA, and D. LEHR. Toxicity studies with the fungicide tetrachloro-para-benzoquinone (Spergon). Jour. Indus. Hyg. & Toxicol. **25**: 98–111. 1943.

52. McNew, George L. Effect of seed treatment on the stand and yield of peas. The Canner (Chicago) **92**(6,7). Jan. 1941.
53. ———— Lima beans' response to seed treatment. The Canner **94**(17). Mar. 28, 1942.
54. ———— Effect of corn seed treated with tetrachloro-para-benzoquinone on the growth and fattening of swine. Iowa State Coll. Jour. Sci. **22**(2): 177–184. 1947–1948.
55. McNew, George L., and H. P. Burchfield. Particle size in relation to the fungitoxicity of dichloronaphthoquinone. Contrib. Boyce Thompson Inst. **16**: 163–176. 1950.
56. Mayer, Fritz. The chemistry of natural coloring matters. (Trans. & rev. by A. H. Cook.) 354 pp. Reinhold Pub. Corp., New York. 1943.
57. Molho, D., and L. Lacroix. L'antagonisme entre la méthyl-2 et la chloro-2-naphtoquinone-1,4 chez *Aspergillus niger*. Compt. Rend. Soc. Biol. **144**: 144–145. 1950.
58. Mulé, F. Antibiotic action of vitamin K. Policlinico (Rome) Sez. Prat. **53**: 653–655. 1946.
59. Nagel, C. M. A new organic fungicide, 2,3-dichloro-1,4-naphthoquinone: Its value as a control for certain defoliation diseases of the tomato. Phytopath. **34**: 1009. 1944.
60. Nord, F. F., Joseph V. Fiore, and S. Weiss. On the mechanism of enzyme action. XXXIII. Fat formation in Fusaria in the presence of a pigment obtained from *Fusarium solani* D_2 purple. Arch. Biochem. **17**: 345–358. 1948.
61. Oxford, A. E. Anti-bacterial substances from moulds. Part V. The bacteriostatic powers of the methyl ethers of fumigatin and spinulosin and other hydroxy-, methoxy-, and hydroxymethoxy-derivatives of toluquinone and benzoquinone. Chem. & Indus. [London] **61**: 189–192. 1942.
62. Page, James E., and F. Arnold Robinson. Polarographic studies. II. Mold metabolites and related quinones. Jour. Chem. Soc. **1943**: 133–135. 1943.
63. Schoene, D. L., H. Douglas Tate, and T. W. Brasfield. Use of quinones as fungicides. Agric. Chem. **4**(11): 24–27, 73, 75–77. Nov. 1949.
64. Schoetensack, Wolfgang. Beeinflussung von Phosphatasen durch Mitosegifte. Naturwiss. **35**: 285. 1948.
65. Schopfer, Wm. H., and Eugene C. Grob. The action of different quinones on the enzymic activity of crystalline urease. Arch. Sci. (Soc. Phys. et Hist. Nat. Genève) **2**: 575–576. 1949. (*Abstr. in* Chem. Abstr. **44**: 6459. 1950.)
66. ———— Vitamin K_3, nicotinic acid and photosynthesis. Arch. Sci. (Soc. Phys. et Hist. Nat. Genève) **2**: 577–580. 1949. (*Abstr. in* Chem. Abstr. **44**: 6488. 1950.)
67. Schreier, O. The injurious action of different quinones on *Planaria gonocephala* D and its relation to Childs' gradient theory. Österreich. Zool. Zeit. **2**: 70–116. 1949. (*Abstr. in* Chem. Abstr. **44**: 749. 1950.)
68. Sexton, W. A. Chemical constitution and biological activity. 412 pp. D. Van Nostrand Co., New York. 1950.
69. Sharvelle, E. G., H. C. Young, Jr., and B. F. Shema. The value of Spergon as a seed protectant for canning peas. Phytopath. **32**: 944–952. 1942.
70. Sleeth, Bailey. The effect of fungicidal seed treatments on guayule seedling emergence. Phytopath. **36**: 999–1010. 1946.
71. Snell, J. M., and A. Weissberger. The reaction of thiol compounds with quinones. Jour. Amer. Chem. Soc. **61**: 450–453. 1939.
72. Spruit, C. J. P., and A. L. Schuiling. Influence of naphthoquinones on the respiration and light emission of *Photobacterium phosphoreum*. Rec. Trav. Chim. **64**: 219–228. 1945. (*Abstr. in* Chem. Abstr. **40**: 5100. 1946.)
73. Suit, R. F., and J. G. Horsfall. A simple method of measuring the interfacial friction of dusted seeds. Phytopath. **29**: 200–204. 1939.
74. Tappi, G., and P. V. Forni. Fungistatic and fungicidal action of heterocyclic derivatives of *o*-benzoquinone. Farmaco Sci. e Tec. (Pavia) **5**: 241–250. 1950.

75. TER HORST, W. P., and E. L. FELIX. 2,3-Dichloro-1,4-naphthoquinone, a potent organic fungicide. Indus. & Eng. Chem. **35**: 1255–1259. 1943.
76. TORDA, CLARA, and HAROLD G. WOLFF. Effect of vitamin K (menadione) on choline esterase activity, acetylcholine synthesis and striated muscle. Proc. Soc. Exp. Biol. & Med. **57**: 236–239. 1944.
77. ———— Effect of 2-methyl naphthoquinone on the action potential of nerve and muscle Amer. Jour. Physiol. **158**: 465–469. 1949.
78. WALKER, J. C., and KARL PAUL LINK. Toxicity of phenolic compounds to certain onion bulb parasites. Bot. Gaz. **96**: 468–484. 1935.
79. WEISS, S., JOSEPH V. FIORE, and F. F. NORD. On the mechanism of enzyme action. XXXVIII. Effect of different naphthoquinones on the fat formation in *Fusarium lini* Bolley. Arch. Biochem. **22**: 314–322. 1949.
80. WELLMAN, R. H., and S. E. A. MCCALLAN. A greenhouse weathering technique for predicting field performance of fungicides. Phytopath. **34**: 1014. 1944.
81. WENDEL, WILLIAM B. The influence of naphthoquinones upon the respiratory and carbohydrate metabolism of malarial parasites. Federation Proc. **5**: 406–407. 1946.
82. WILCOXON, FRANK, and S. E. A. MCCALLAN. Fungicidal action of organic thiocyanates, resorcinol derivatives, and other organic compounds. Contrib. Boyce Thompson Inst. **7**: 333–339. 1935.
83. WINGARD, S. A., and E. T. BATTEN. Treat seed peanuts for profit. Virginia Agric. Exp. Sta. Bull. 382. 11 pp. 1945.
84. WOOLLEY, D. W. Observations on antimicrobial action of 2,3-dichloro-1,4-naphthoquinone, and its reversal by vitamins K. Proc. Soc. Exp. Biol. & Med. **60**: 225–228. 1945.

CHROMATE COMPLEXES AS FUNGICIDES[1]

F. R. Whaley and J. B. Harry[2]

Normal chromates such as silver chromate (8) and copper chromate (4) have been reported as being fungicidal, but they are too phytotoxic for practical use as foliage fungicides. The chromate complexes described herein differ from the normal chromates in that they are basic and irregular in their stoichiometry. They usually involve more than one metal (besides chromium) in the complex, and frequently the observed biological effect is greater than that predicted from the known toxicity of the ingredients. The present report describes the development of some interesting fungicides from the general class of chromate complexes.

SOLUBILITY BEHAVIOR OF THE CHROMATE COMPLEXES

Attempts to determine the solubility of complex basic chromates disclosed that they do not dissolve in water as a unit, but that water acts on them to change their composition. Table I shows how the dissolved Cr

TABLE I

Relation of Solubility to Suspension Concentration

Chromate complex	Solubility in g. per 100 ml. at indicated suspension concns. (g./100 ml.)				
	0.1	1.0	10	30	50
Zn—Cr {Cr(VI)		0.0013	0.0066	0.0105	
Zn		0.0014	0.0050		
Zn—Fe—Cr {Cr(VI)	0.0027	0.0151	0.0900	0.1950	0.2600
Zn		0.0110	0.0400		
Cu—Zn—Cr Cr(VI)		0.00074	0.00537		

(VI) and Zn in typical basic chromates vary with suspension concentration. The solubility of both metals increases with increasing suspension concentration but the increase in solubility is not directly proportional to the increase in concentration of the suspension, as would be expected of a normal material below its saturation concentration. Furthermore, the molar ratios of the complex in solution differ from that of the original solid and vary with the amount of dilution. This is shown in Table II. Obviously

[1] Invitational paper presented before the American Chemical Society in Philadelphia, Pa., on April 12, 1950.

[2] Union Carbide and Carbon Corporation, New York, N. Y.

TABLE II

MOLAR RATIO OF ZINC TO CHROMIUM IN SOLID AND SOLUTION

Complex	In orig. solid	Molar ratio in solution at indicated concns., g. per ml.	
		1	10
Zn—Cr	4.04	0.86	0.60
Zn—Fe—Cr	2.46	0.58	0.35

the composition of the solid residue differs somewhat from that of the solid before the solubility test. While this difference may be ignored in the case of a complex of low solubility, in some cases it is sufficient to introduce practical difficulties in preparing a desired complex by a process requiring a large amount of washing.

Apparently a true equilibrium is eventually obtained when a given amount of the complex is shaken sufficiently in water. Reference to Figure 1 shows the relation between the Cr(VI) solubility and the concentration

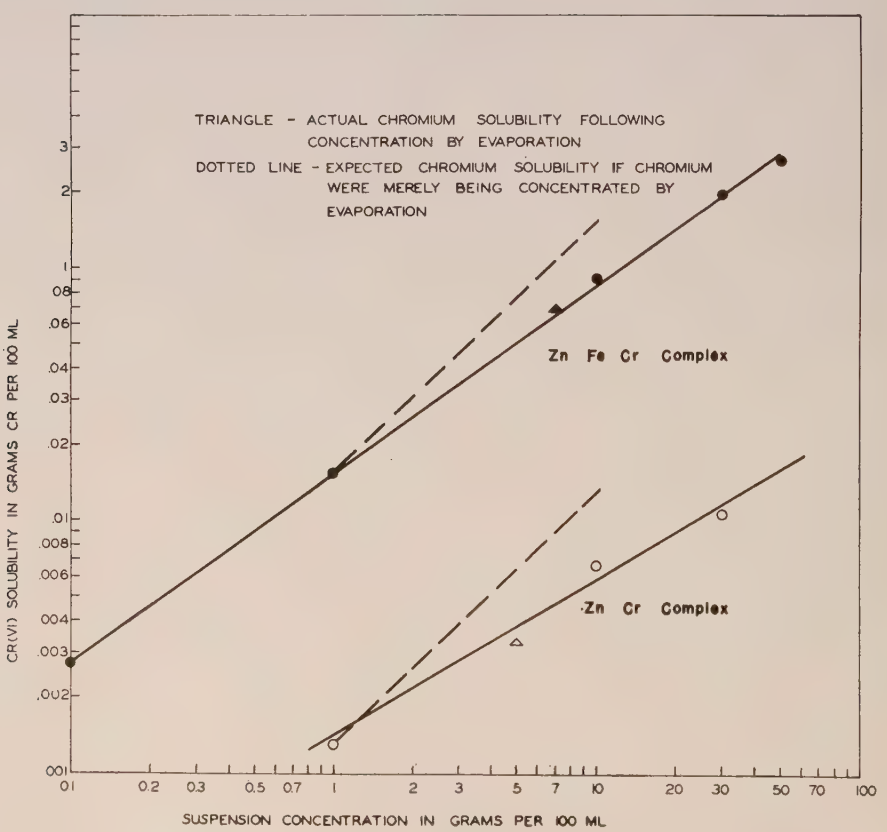


FIGURE 1. Relation between Cr(VI) solubility and suspension concentration.

of the suspension (solid lines) for two of the complexes. On log-log coordinates the relation is nearly a straight line over a wide concentration range. The circles show the solubilities actually obtained when starting with the suspensions indicated on the abscissas. The reversibility of this relationship was shown by vaccum evaporation of suspensions initially containing 1 gram of solid per 100 milliliters. If the Cr(VI) in solution from the original suspension were merely concentrated during the evaporation, its solubility should be directly proportional to the suspension concentration (dotted line). On the other hand, if the Cr(VI) in solution comes only from a normal chromate in a saturated solution, concentration should not change its solubility. The actual solubilities determined after vacuum evaporation followed neither of these courses, but were found to lie on the respective solubility lines for the complexes (triangular points). Thus each complex reacts with water reversibly to form a series of hydrolysis products having a composition which varies continuously (known as non-Daltonian compounds or Berthollides) and in which obedience to stoichiometric atomic ratios may be only accidental.

In determining and comparing the solubilities of a large number of experimental complexes it is convenient to select a standard suspension concentration. For this purpose in these studies 1 gram of solid per 100 milliliters of water was chosen as the standard suspension. This is equivalent to about 8.3 pounds per 100 gallons, or near the maximum that might be used in customary spray techniques. In correlating the biological test results with the standard Cr(VI) solubilities of several hundred basic chromates, it was concluded that at Cr(VI) solubilities under 0.0001 gram per 100 milliliters the chromium contributes little or no fungicidal action, and above 0.03 gram per 100 milliliters the chromium causes some phytotoxicity.

SYNERGISM OF Cr(VI) WITH Cu(II) AND WITH Zn

Using the spore-inhibition technique (1,2) the basic chromates were tested to determine the effective dose needed to inhibit 50 per cent spore germination (ED50). It was found that once the lower solubility limit is exceeded, so that a minimum concentration of dissolved Cr(VI) is available, further increase in Cr(VI) solubility has relatively little effect on fungicidal action as compared with the effect of the amount of reserve Cr(VI) available, expressed as the percentage of CrO_3 in the complex.

The ED50 was determined for each of 15 chromate complexes of suitable solubility containing no other fungicidal component. Since these contained varying amounts of other materials, which may be considered as vehicles for the CrO_3, the ED50 for each was multiplied by its respective fraction of CrO_3 to obtain the dose of CrO_3 needed to obtain 50 per cent spore inhibition independent of the rest of the complex. The average for 15 chromates tested on four fungus diseases was $ED50_{CrO_3} = 0.32$ where

Cr(VI) was the sole fungicidal component present. A similar calculation from test results on various copper fungicides devoid of other known fungicidal elements showed an average $ED50_{CuO} = 3.16$. Similarly $ED50_{ZnO} = 18.2$. In the laboratory tests, therefore, Cr(VI) was more fungicidal than either Cu(II) or Zn.

In case the complex contains both Cr(VI) and Cu(II) (but no other fungicidal components) the quantity $ED50_T$ is defined as $ED50 \times \frac{\%\ CrO_3 + \%\ CuO}{100}$, which represents the dose of combined toxic ingredients giving 50 per cent spore inhibition, with the balance of the complex again considered merely a vehicle.

$$\text{Let } W_{CrO_3} = \frac{\%\ CrO_3}{\%\ CrO_3 + \%\ CuO}, \quad \text{and} \quad W_{CuO} = \frac{\%\ CuO}{\%\ CrO_3 + \%\ CuO}.$$

The W terms represent the proportion of each toxic component relative to the total toxic components, irrespective of vehicle. Bliss (3) has shown that the most effective joint action of a combination of toxic ingredients, which is predictable from their separate effects, is similar joint action. His formula applied to the present case is:

$$\frac{W_{CrO_3}}{ED50_{CrO}} + \frac{W_{CuO}}{ED50_{CuO}} = \frac{1}{ED50_T},$$

or

$$\frac{\%\ CrO_3}{ED50_{CrO_3}} + \frac{\%\ CuO}{ED50_{CuO}} = \frac{\%\ CrO_3 + \%\ CuO}{ED50_T},$$

or

$$\frac{\%\ CrO_3}{0.32} + \frac{\%\ CuO}{3.16} = \frac{100}{ED50}.$$

The left hand terms depend upon the composition of the complex and some previously determined constants. The right hand term is capable of direct measurement for any complex. If the observed toxicity is greater than that calculated from the left hand terms, the toxicity is greater than additive and synergism is in evidence.

Table III shows 21 copper chromate complexes listed in order of increasing W_{CrO_3}. The difference between the observed and calculated toxicity is given in the next to the last column. In the last column rank numbers are assigned to the differences (without regard to algebraic sign), and the rank numbers are given the same sign as the differences to which they

TABLE III

DATA SHOWING SYNERGISM OF CHROMATES WITH COPPER AS CALCULATED BY METHOD OF BLISS

CrO_3, %	CuO, %	Sum	W_{CrO_3}	W_{CuO}	W_{CrO_3} 0.32	W_{CuO} 3.16	$\frac{1}{ED_{50T}}$ Calcd.	$\frac{1}{ED_{50T}}$ Observed	Difference +	Difference −	Rank +	Rank −
8.4	46.8	55.1	0.152	0.848	0.48	0.27	0.75	1.15	0.40		7	
11.1	42.8	53.5	0.208	0.792	0.65	0.25	0.90	2.86	1.96			
11.2	39.4	50.7	0.222	0.778	0.70	0.25	0.95	2.44	1.49			
20.2	64.1	84.3	0.239	0.761	0.75	0.24	0.99	1.96	0.97			
23.1	64.3	87.4	0.264	0.736	0.83	0.23	1.06	1.33	0.27		4	
6.6	16.9	23.4	0.280	0.720	0.88	0.23	1.11	5.00	3.89			
25.5	60.7	86.2	0.295	0.704	0.92	0.22	1.14	1.82	0.68			
21.9	38.8	60.7	0.301	0.639	1.13	0.20	1.33	1.04		−.29		−5
36.5	45.7	82.2	0.444	0.556	1.39	0.18	1.57	2.94	1.37			
26.8	32.5	59.2	0.451	0.549	1.41	0.17	1.58	1.78	0.20		3	
30.7	36.8	67.5	0.455	0.545	1.42	0.17	1.59	1.47		−.12		−1
36.2	42.9	79.1	0.458	0.542	1.43	0.17	1.60	1.41		−.19		−2
38.4	41.3	79.7	0.482	0.518	1.51	0.16	1.67	4.00	2.33			
31.9	29.8	61.7	0.516	0.484	1.61	0.15	1.76	3.00	1.24			
30.1	26.4	56.6	0.533	0.467	1.67	0.15	1.82	3.20	1.38			
42.3	34.9	77.2	0.547	0.453	1.71	0.14	1.85	1.31		−.54		−8
31.6	21.1	52.7	0.60	0.40	1.88	0.13	2.01	11.1	9.00			
34.5	22.0	56.5	0.61	0.39	1.91	0.12	2.03	2.33	0.30		6	
34.5	15.2	49.7	0.695	0.305	2.17	0.10	2.27	15.4	13			
33.9	13.9	47.8	0.709	0.291	2.22	0.09	2.31	20	18			
39.2	7.4	46.6	0.842	0.158	2.63	0.05	2.68	4.00	1.32			
												$T=-16$

For significance at $P=0.01$, T must be under 43.

correspond. Wilcoxon's (10) statistical T test for significance is then applied. The sum of the negative rank numbers is considerably under 43, which is the T value corresponding to $P=0.01$ with 21 replicates. This means the evidence for synergism is very definite; the probability of such results occurring by chance is less than 0.01.

Table IV shows similar data for zinc chromates. The data are insuffi-

TABLE IV

SYNERGISM OF CHROMATES WITH ZINC

CrO_3, %	ZnO, %	Sum	W_{CrO_3}	W_{ZnO}	W_{CrO_3} 0.32	W_{ZnO} 18.2	$\frac{1}{ED_{50T}}$ Calcd.	$\frac{1}{ED_{50T}}$ Observed
17.9	70	87.9	0.20	0.80	0.63	0.04	0.67	1.14
20	67.3	87.3	0.23	0.77	0.72	0.04	0.76	3.00
14.6	29.0	43.6	0.34	0.66	1.06	0.04	1.10	2.70
15.1	50.2	65.2	0.23	0.77	0.72	0.04	0.76	2.40
15.6	50.4	66.0	0.24	0.76	0.75	0.04	0.79	1.52

cient to apply the Wilcoxon statistical test, but synergism again appears to be quite definite. The toxicity $\left(\frac{1}{ED_{50T}}\right)$ of both copper and zinc chromates are plotted on log-log coordinates in Figure 2. The solid lines show the toxicity calculated from similar joint action and the circles show the values actually obtained.

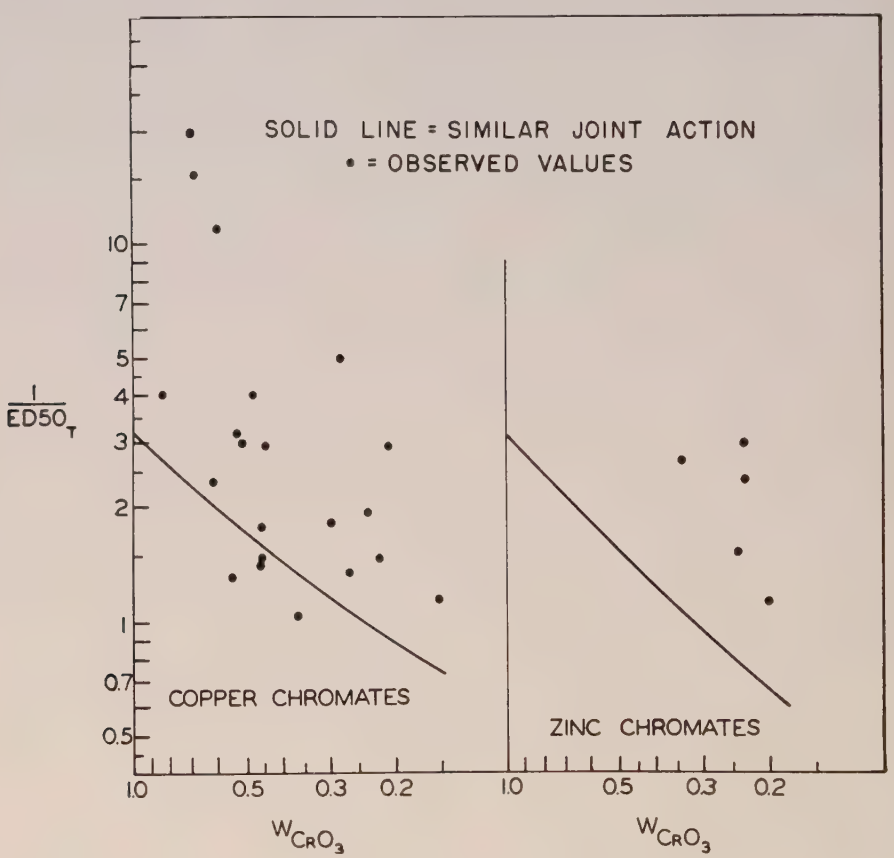


FIGURE 2. The fungitoxicity of chromates containing copper and zinc.

RESULTS OF EARLY FIELD TESTS

The encouraging laboratory results were next confirmed in a qualitative sense in greenhouse tests and finally an extensive field-test program was inaugurated. Field tests were conducted over a period of years on potatoes (*Solanum tuberosum* L.) for control of late blight and early blight. The chromate complexes were compared with Bordeaux mixture (8-8-100, 8 lb. of $CuSO_4 \cdot 5H_2O$ and 8 lb. of lime per 100 gal.) and several representative

fixed coppers containing at least 50 per cent copper. Usually a representative of the dithiocarbamates was also included. These tests revealed that in blight control the chromates without copper are not so effective as Bordeaux, a result which contradicted the laboratory results. Basic copper chromate complexes, however, are better in blight control than Bordeaux, thus confirming the synergism found in laboratory tests. Several types of chromates appeared promising in these tests with respect to both fungus and insect control, with a copper-zinc chromate (No. 169) emerging in 1940 as equal to or superior to commercial treatments. Part of these data are summarized in Table V. In order to repel insects as well as Bordeaux

TABLE V

YIELD INCREASE OF POTATOES SPRAYED WITH THREE DIFFERENT FUNGICIDES, WITH AND WITHOUT DDT

Fungicide	Yield in per cent of check	
	Without DDT	With DDT
State College, Pa. and Huttonsville, W. Va., 1946		
Bordeaux, 8-8-100	152	168
Nabam,* 2 qt. per 100 gal.	141	173
169, 6 lb. per 100 gal.	167	Not tested
Belle Glade, Fla., 1945		
Fixed copper, 4 lb. per 100 gal.	128	141
Nabam,* 2 qt. per 100 gal.	137	156
169, 5.5 lb. per 100 gal.	147	152
State College, Pa., 1946		
Fixed copper, 4 lb. per 100 gal.	132	147
9, Basic copper chromate, 2 lb. per 100 gal.	Not tested	132
169, 6 lb. per 100 gal.	142	Not tested

* Sodium ethylenebisdithiocarbamate, used with zinc sulfate.

mixture, 169 was developed with a high zinc oxide content (5). In the absence of DDT, 169 compared very favorably with commercial products, but evidence from nationwide tests indicated that DDT would be accepted as a standard potato insecticide to be used in conjunction with a fungicide. For this reason the high zinc oxide content of 169 appeared to be no longer necessary. A basic copper chromate (No. 9) was inferior to copper-zinc chromate in blight control (DDT present with No. 9) showing that some zinc oxide was necessary in the complex to give the best fungicidal results irrespective of its insect-repellent properties. This serves as field confirmation of the zinc-chromium synergism found in laboratory tests.

DEVELOPMENT OF COPPER-ZINC CHROMATES

Several copper-zinc chromates were prepared. The compositions in mole fractions of the principal ingredients are shown in Figure 3 for compounds 169, 658 and 640, as well as for basic copper chromate 9 and basic zinc chromate 181. The compositions are also shown in the first column of

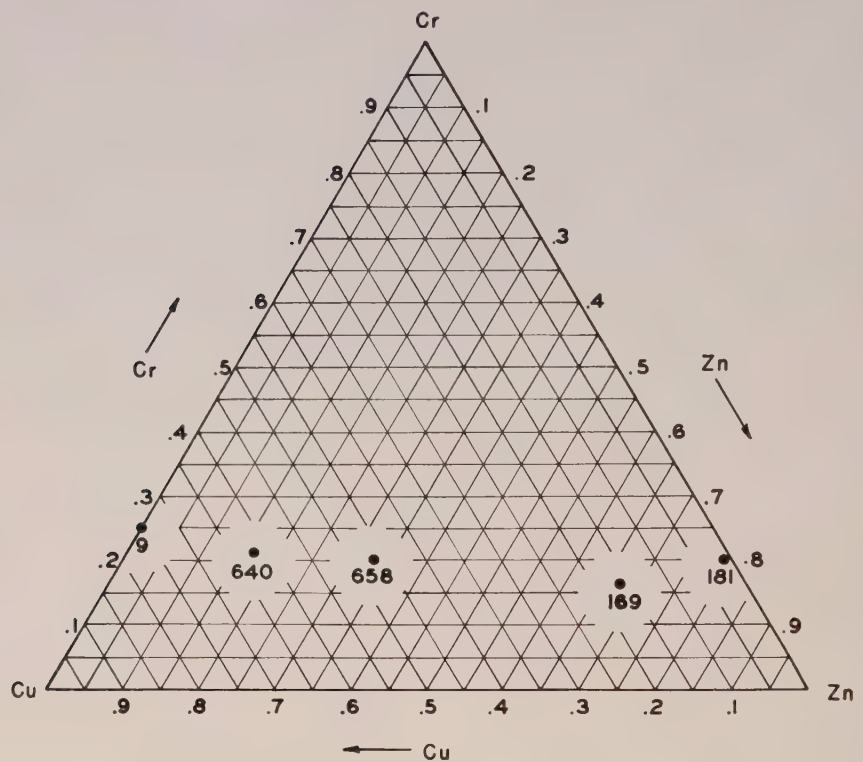


FIGURE 3. Mole fraction of copper, zinc, and chromium in copper-zinc chromates.

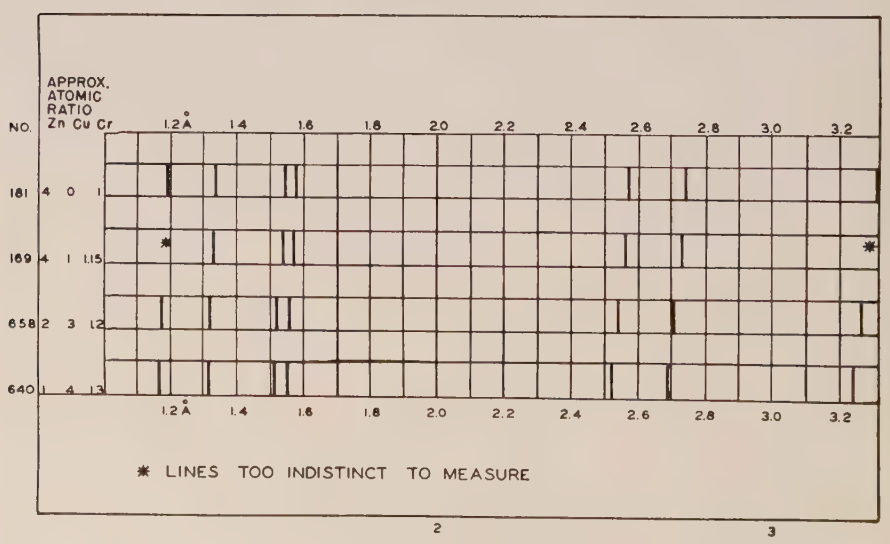


FIGURE 4. Interplanar distances "*d*" (diagrammatic). Partial pattern for basic zinc chromate and copper-zinc chromate.

TABLE VI

YIELD AND DISEASE CONTROL OF POTATOES SPRAYED WITH FOUR DIFFERENT FUNGICIDES AND DDT, FLA. 1947

Fungicide	Yield, lb. per plot	Late blight, % leaves killed	Early blight, % spotted leaves
169, 4 lb. per 100 gal.	62	15	Trace
658, 2 lb. per 100 gal.	65	10	Trace
Fixed copper, 5 lb. per 100 gal.	56	15	10
Zineb,* 2 lb. per 100 gal.	61	25	15

* Zineb is zinc ethylenebisdithiocarbamate.

Figure 4 which gives a diagrammatic partial X-ray pattern for each. The principal diffraction lines corresponding to interplanar distances *d* are displaced on replacing zinc with copper, but the basic pattern remains the same, even with 80 per cent replacement as in 640. At 90 per cent replacement the pattern of basic copper chromate 9 is superimposed on the pattern of copper-zinc chromate. Both 658 and 640 were tested in the field, and 658 was believed to be better. The 1947 Florida field tests are summarized in Table VI where 658 at 2 pounds per 100 gallons is equivalent to 169 at 4 pounds per 100 gallons and both are better than two standard fungicides.

The copper-zinc chromates were tested more extensively in the summer of 1947 as shown in Table VII. In five of the six tests, the copper-zinc chromate was superior to the average of the two standard fungicides used for comparison. Since 658 gave satisfactory performance at half the dosage of 169, the former was chosen for further development.

The 1948 and 1949 tests were on a still wider scale, but due to very low disease potential, average comparisons are not very reliable. In areas where some disease was observed, 658 maintained its position as a superior potato fungicide.

Some interesting dust residue tests were made in Ohio comparing 658 dust (6 per cent 658, 6 per cent DDT, 88 per cent talc) with a fixed copper fungicide (10 per cent fixed copper, 6 per cent DDT and 84 per cent talc).

TABLE VII

PER CENT YIELDS OF POTATOES SPRAYED WITH THREE DIFFERENT FUNGICIDES WITH DDT IN SIX AREAS

Fungicide	Yields as per cent of check yield						
	Pa., 1	Pa., 2	W. Va.	Ohio, 1	Ohio, 2	Ohio, 3	Av.
Bordeaux	130	112	94	125	135	127	121
Nabam*	139	121	85	134	148	132	127
Cu—Zn—Cr	135**	119**	104**	146†	160†	125†	132

* Sodium ethylenebisdithiocarbamate, 2 qt. per 100 gallons plus zinc sulfate.
** 658, 2 lb. per 100 gal.
† 169, 4 lb. per 100 gal.

The fixed copper contains 53 per cent copper, while 658 contains 30 per cent copper. Each formulation was applied at 50 pounds per acre. Shortly after each application, leaf samples were analyzed for copper, and the results in milligrams of metallic copper per 20 g. of leaves were recorded as deposit. Just before the next application, similar samples were analyzed for copper and recorded as residue. These were averaged over the season for two areas to give the values shown in Table VIII. The ratio of average

TABLE VIII

PERSISTENCE OF COPPER ON LEAVES DUSTED WITH 658 OR FIXED COPPER

Dust	Lb. Cu per acre	Mg. Cu per 20 g. leaves*		
		Deposit	Residue	Tenacity, $\frac{\text{residue}}{\text{deposit}}$
658, 6%	0.90	2.34	0.58	0.25
Fixed Cu, 10%	2.65	3.91	1.11	0.28

* Seasonal average of tests in two areas.

residue to average deposit is defined as tenacity. The tenacities appear to be in the same order of magnitude. The copper deposited by the fixed copper is obviously greater than that deposited by 658, but not as much greater as would be expected from the higher copper content and higher dosage of the fixed copper.

The data may be expressed in another form to show the relative tendency of the two materials to build and maintain a deposit on the foliage. Equating to unity the copper applied on a given area as fixed copper, the copper applied on the same area as 658 would be 0.34 (from the product analyses and dosages). However, the ratios of the copper actually deposited as 658 to that deposited as fixed copper is 0.66. This shows that 658 tends to build better deposits at the time of dusting than does the fixed copper fungicide. The copper in the residue is also relatively greater for 658 than for the fixed-copper fungicide after weathering, 0.52.

OTHER COMPLEX CHROMATE FUNGICIDES

A calcium-cadmium-copper-zinc chromate 531 has been developed as a turf fungicide, which is particularly effective in the control of dollar spot on bent grasses (9). In spite of the imposing number of active components, evidence supports the view that this is a chemical complex rather than a mixture of oxides, hydroxides, or salts. Part of this evidence is based on X-ray studies of the components and intermediate products as shown diagrammatically in Figure 5. An X-ray spectrometer equipped with a Geiger counter was used to determine the diffraction angles of the diffraction lines over the range indicated. The heights of the vertical lines are

proportional to the heights of the peaks in the respective spectrometer charts. Such a pattern serves as a thumb-print to identify crystalline materials alone or in mixtures. The principal diffraction lines of the ingredients are shown first. Pattern A shows the pattern of the yellow-brown reaction product of cadmium oxide and chromic acid. Patterns B and C show the intermediate products after copper sulfate pentahydrate and zinc oxide are added, respectively. At this point the intermediate is rather dark brown. After the addition of lime the color changes through gray-green and olive-

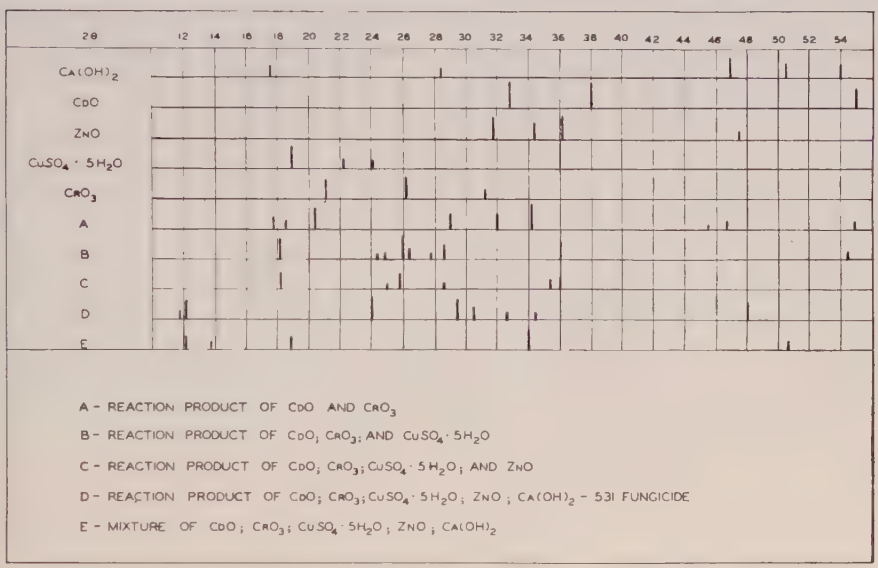


FIGURE 5. Diagrammatic representation of the X-ray patterns of the ingredients of the calcium-cadmium-copper-zinc chromate complex before and after reaction.

green to yellow-green, the color of the final product (Pattern D). It will be noted that the X-ray pattern of the final product is as unlike that of the ingredients and intermediates as is its color. None of the principal lines of ingredients or intermediates appear in the pattern of the product. Pattern E is obtained from a mixture of the reactants which had been previously ground separately. In an attempt to avoid reaction, dry materials were mixed without grinding before the X-ray pattern was made. In spite of these precautions, some reaction evidently occurred, as is evidenced by the appearance of the product line at $2\theta = 12$ and the disappearance of lines from the components except those of lime.

A mercury-zinc chromate has lately shown promise as a seed treatment particularly on corn (6, 7). In some demonstration plots in Iowa it gave considerably larger yields than commercial treatments. The entire series of mercury-zinc chromates appears to be similar to other series studied in that

a gradation of properties results from a gradation of composition and within certain composition limits non-Daltonian compounds or Berthollides exist.

In general the complex basic chromates are easy to handle and noncorrosive to containers and spraying equipment. They comprise a class of materials interesting not only for their biological action but also for their physical and chemical properties.

Two of the fungicides described herein, a potato fungicide (658) and a turf fungicide (531), are being produced commercially under the Crag trade-name and others are receiving extensive field tests. These materials are examples of synthetic inorganic materials which take their places alongside the excellent synthetic organic fungicides developed during the past few years, and serve to illustrate the role which may be played by new and unusual inorganic compounds in the field of agricultural chemicals.

ACKNOWLEDGMENTS

The authors wish to express appreciation for the excellent help rendered throughout the project by Dr. R. H. Wellman of the Biological Research Department, Carbide and Carbon Chemicals Division, Union Carbide and Carbon Corporation. They also take this opportunity to acknowledge the indispensable aid of the cooperators who conducted confirmatory field tests, particularly Dr. H. W. Thurston, Jr. of Pennsylvania State College, Dr. J. D. Wilson of the Ohio Agricultural Experimental Station, and Dr. J. G. Leach of the University of West Virginia.

LITERATURE CITED

1. AMERICAN PHYTOPATHOLOGICAL SOCIETY. COMMITTEE ON STANDARDIZATION OF FUNGICIDAL TESTS. The slide-germination method of evaluating protectant fungicides. Phytopath. **33**: 627–632. 1943.
2. ———— Test tube dilution technique for use with the slide-germination method of evaluating protectant fungicides. Phytopath. **37**: 354–356. 1947.
3. BLISS, C. I. The toxicity of poisons applied jointly. Ann. App. Biol. **26**: 585–615. 1939.
4. CHAVASTELON, R. Botanique Appliquée—Sur un procédé de conservation des bois. Compt. Rend. Acad. Sci. [Paris] **176**: 1178–1179. 1923.
5. HARRY, J. B., R. H. WELLMAN, F. R. WHALEY, H. W. THURSTON, JR., and W. A. CHANDLER. Development of copper-zinc-chromate complexes as potato fungicides. Contrib. Boyce Thompson Inst. **15**: 195–210. 1948.
6. MCCALLAN, S. E. A. Evaluation of chemicals as seed protectants by greenhouse tests with peas and other seeds. Contrib. Boyce Thompson Inst. **15**: 91–117. 1948.
7. ————. Some chromate complexes and organic compounds as seed protectants. Contrib. Boyce Thompson Inst. **16**: 5–20. 1950.
8. NIELSEN, LOWELL W. Studies with silver compounds and mixtures as fungicidal sprays. New York [Ithaca] Agric. Exp. Sta. Mem. 248. 44 pp. 1942.
9. THURSTON, H. W., JR. Cadmium is base of new method of dollar spot control. Golfdom pp. 70–71; 92–93. June, 1948.

WILCOXON, FRANK. Some rapid approximate statistical procedures. (rev.) American Cyanamid Co. 1949.

GERMINATION OF SEEDS OF JUNIPERUS VIRGINIANA L.

LELA V. BARTON

Juniperus virginiana L. is one of the most valuable forest trees in the United States. The reforestation program of much badly depleted forest land has been seriously hampered because of the difficulty and cost involved in producing seedlings and in transplanting them to the forest.

It has been reported (1, 3, 4, 5, 6, 7, 8) that low temperature pretreatment (stratification) is necessary for germination of the seeds. Some of the investigators have also indicated that part of the difficulty is due to the seed coat. Chadwick (3) removed the waxy coats by soaking several hours in alcohol or by warm water treatment, while Webster and Ratliffe (8) soaked the seeds for 20 minutes in luke-warm sodium-lye solution before stratifying them at low temperatures. Parker (7) found that scarification of the seeds resulted in earlier germination but did not increase final germination.

In addition to the stratification requirement of these seeds, they must have a fairly low temperature for the germination process itself. Chadwick (3) states that germination after stratification is best at 50° to 55° F., and that it is considerably retarded at temperatures above 65° to 70° F. Pack (6) also noted that after-ripened seed should be planted at a temperature below 15° C. (59° F.).

The seeds, one to three each, are borne in a berry-like fruit formed of fleshy coalescent scales of the fertile catkins. The pulp and skin of these fruits have been reported to reduce germination. Afanasiev and Cress (1) found that stratification of seed without removing the pulp hindered after-ripening and that germination of such seed after three months of stratification was only 10 per cent as compared with 30 per cent for seed cleaned prior to stratification.

In spite of the work which has been done on the germination of red cedar, nurserymen experience difficulty in producing seedlings. In practice, only a few seeds germinate the first year. Most of them germinate the second year after planting, and some the third year. There is need for more experimental work to determine how to treat the seeds so that they will germinate in a predictable and uniform manner. Furthermore, this treatment should require a short time so as to enable one to reseed within the year in case of germination failure, thus ensuring a constant supply of seedlings for planting projects and reforestation programs. If possible, also, planting of berries is desirable, thus eliminating the difficult and expensive task of cleaning the seeds. The present tests were undertaken in

Copyright, 1952, by Boyce Thompson Institute for Plant Research, Inc.

an effort to determine more specifically the germination requirements of these seeds, with the hope that such studies would lead to a simplified, satisfactory method of seedling production on a large scale.

MATERIAL AND METHODS

Berries and seeds used for the tests were collected near Anselmo, Neb. during December 1948 and were furnished through the courtesy of Mr. R. K. Smith, Forest Supervisor, Nebraska National Forest, Halsey, Neb., at whose suggestion the present investigation was undertaken.

Many of the seeds were small and empty. Consequently, all clean seeds were sieved and the smallest ones removed before starting germination tests. Four lots of 100 seeds each of the sieved seeds were cut open to determine whether they were filled with apparently healthy embryo and endosperm. These lots were found to contain 82, 77, 87, and 82 per cent, with an average of 82 per cent filled seeds. Three lots of 25 embryos each were excised and placed on moist filter paper in the laboratory for viability tests. After five days, 76 per cent of them appeared firm and good, the remainder being soft and discolored. Thus the germination to be expected from the lot of cleaned seeds was about 62 per cent.

Germination tests were also made using berries. Three lots of 50 berries each were found to contain 94, 99, and 95 seeds. Twenty-six per cent of these seeds were empty. Because the small seeds could not be screened out in this case, as for the clean seeds, a somewhat smaller percentage germination could be expected.

Both berries and seeds were planted in soil directly and after pretreatment in various ways. The soil mixture used was composed of sod soil, sand, and granulated peat moss in equal parts. When outside temperatures permitted, the temperature of the greenhouse was kept at 20° C.

RESULTS AND DISCUSSION

Temperature requirement for germination. Two hundred seeds each were mixed with moist granulated peat moss and placed at constant temperatures of 15°, 20°, 25°, and 30° C., and daily alternating temperatures (5 days each week) of 10° to 20°, 10° to 30°, 15° to 30°, and 20° to 30° C., where 3, 1, 0, 0, 3, 10, 7, and 0 per cent germination respectively was secured, during a period of four and one-half months. This confirmed the relatively low temperature required for germination of these seeds and emphasized the need for pretreatment for complete germination.

Pretreatment. Some seeds are known to possess an impermeable coat together with a dormant embryo. One of the best known ways to break the dormancy of temperate-zone seeds is to give them a period in a moist medium at low temperature (stratification) before planting. The afterripening necessary to break the dormancy takes place only after the seeds

have imbibed water. Thus, if there is an impermeable seed coat, it must be made permeable before the low temperature treatment will be effective. This fact, coupled with favorable effects of seed coat treatments of *Juniperus* obtained by other workers (3, 7, 8), determined our line of attack on the problem.

The use of concentrated sulphuric acid to remove hard coat effects is well known. Also, it has been demonstrated that a period at high temperature in a moist medium is effective in rendering seed coats permeable (2).

TABLE I

EFFECT OF PRETREATMENT OF JUNIPERUS VIRGINIANA SEEDS IN MOIST GRANULATED PEAT MOSS ON SEEDLING PRODUCTION IN SOIL IN THE GREENHOUSE. 2×100 SEEDS EACH

Pretreatment			Per cent seedling production after storage at low temperature		
Sulphuric acid	Weeks at 25° C.	Low temp., ° C.	1 mo.	2 mo.	3 mo.
None	None	1	0	3	11
		5	0	17	34
		10	0	1	2
None	2	1	0	17	47
		5	2	28	58
		10	0	0	2
None	4	1	0	24	59
		5	4	53	77
		10	0	0	0
None	8	1	1	15	43
		5	4	54	76
		10	1	1	1
30 min.	None	1	1	17	34
		5	2	37	63
		10	2	0	1

This latter method seemed more promising from a practical point of view and hence was investigated in greater detail. Some of the results are shown in Table I. Clean seeds were used for these tests. For the pretreatment at both high and low temperatures the seeds were held in moist granulated peat moss at controlled temperatures. After the pretreatment period, samples were planted in soil in the greenhouse. Intact seeds were given 0, 2, 4, 8, and 16 weeks at 25° C. preceding 1, 2, 3, and 4 months at 1°, 5°, 10°, and 15° C. The data for 16 weeks at 25° C. and 4 months at 1°, 5°, and 10° C. have been omitted because they show no advantage over shorter periods at the same temperatures. Fifteen degrees C. was totally ineffective as an after-ripening temperature and so does not appear in the

table. One lot of seeds soaked in concentrated sulphuric acid for 30 minutes and receiving no high temperature pretreatment is also included in Table I. Untreated controls gave no germination in soil in the greenhouse.

Up to 34 per cent germination was obtained after three months at 5° C. without preceding exposure to 25° C. This figure corresponds approximately to that most often found in the literature and probably represents the limit of germination from seeds so treated. This means that the seed coats vary in degree of impermeability. Certain of them permit water absorption and allow after-ripening to proceed at the low temperature. Others require some treatment before the low temperature can have any effect. It will be noted that as short a time as two weeks at 25° C. had sufficient effect on the seed coats to increase seedling production after a subsequent period of three months at 5° C. from 34 to 58 per cent. An extension of the time at 25° C. to four or eight weeks further increased germination to 77 and 76 per cent, respectively. From the observations on the number of filled seeds and the viable embryos in the lot described above, this represents complete germination. One degree C. was less effective than 5° C. for after-ripening and 10° C. was totally ineffective. It should be kept in mind that the figures in Table I represent actual seedling production in a greenhouse with a minimum temperature of 20° C. The seed samples were planted in the greenhouse from March 3 to June 30. For example, seeds receiving four weeks at 25° C. followed by three months at 5° C. were planted in the greenhouse on June 3, and those receiving eight weeks at 25° C. followed by three months at 5° C. were planted in the greenhouse on June 30. This means, then, that with the proper pretreatment seeds will germinate to form good seedlings at temperatures found by many, including this laboratory, to completely inhibit germination of untreated seeds. This alone is of great practical value. A longer period than three months at 5° C. is not feasible, for germination begins at the low temperature itself about that time, and young seedlings are apt to be injured in planting.

That a period at 25° C. functions by removing impermeable coat effects is shown by the successful replacement of such a period by the use of concentrated sulphuric acid (Table I). Complete germination is secured after three months at 5° C. provided the seeds were soaked for 30 minutes in concentrated sulphuric acid before placing in moist granulated peat moss. The volume of acid should be about three times that of the seeds used. At the end of the soaking period, the acid should be drained from the seeds which should then be plunged immediately into a large amount of cold water to prevent heating. They should then be washed thoroughly in running water, and preferably rubbed gently to remove some of the carbonized surface and any remaining acid before placing in the germination medium. Sulphuric acid treatment saves the two to eight weeks at

25° C. required to produce the same results, but the latter method is easier and safer for the ordinary person. The soaking time in the acid can be extended to one hour without damage to the seeds.

Direct seeding in soil. Nurserymen and foresters are interested in a method for direct seeding of *Juniperus*. Also, the use of berries instead of clean seeds for planting would mean a considerable saving of time and money. With these objectives in mind, both berries and cleaned seeds, with and without sulphuric acid treatment, were planted in soil in flats. Sample flats were kept in the greenhouse for 0, 1, 2, and 4 months before transferring to a 5° C. room for 1, 2, 3, and 4 months. After the period at 5° C. the flats were again placed in the greenhouse where seedlings appeared (Table II).

TABLE II

SEEDLING PRODUCTION IN THE GREENHOUSE FROM JUNIPERUS VIRGINIANA SEEDS GIVEN SPECIAL TEMPERATURE TREATMENT AFTER PLANTING IN SOIL IN FLATS. 2×50 BERRIES; 2×100 SEEDS

	Months in greenhouse preceding period at 5° C.	Per cent* seedling production after months at 5° C.			
		1	2	3	4
Berries	None	0	3	8	10
	1	1	14	27	32
	2	3	25	34	41
	4	3	18	49	48
Seeds	None	0	19	48	46
	1	6	36	68	57
	2	14	36	68	71
	4	7	30	64	64
Seeds in H_2SO_4 for 30 min.	None	5	56	73	69

* For berries the actual numbers of seedlings produced, rather than per cent, are given.

Again, very satisfactory germination of clean seeds was secured when the flats received 1, 2, or 4 months at greenhouse temperature followed by at least three months at 5° C., and again sulphuric acid treatment replaced the need for the initial period at high temperature. Seeds in the berries responded to the same sort of treatment as clean seeds. Since examination of the berries showed that 100 of them contained approximately 192 seeds, and that only 56 per cent of these could be expected to germinate, about 108 seedlings would be the maximum which could be expected. Table II shows less than half that number for the most favorable conditions.

Although the effectiveness of pretreatment methods have just been shown for soil plantings, temperatures were still imposed under controlled conditions, i.e. greenhouse and 5° C. room, which would not be available

for most people. For smaller lots of seeds, of course, it would be possible to plant in flats which should be watered and kept in any warm place for two to eight weeks and then transferred out-of-doors under conditions which would give at least three months of 1° to 5° C. With this procedure seedlings could be obtained the first spring after harvest of the seeds.

Effect of time of planting out-of-doors. The ideal planting condition from a practical point of view, would be direct seeding in the nursery or field. This can be secured very easily in a region with the climate of Yonkers, N. Y., as is shown in Table III. Plantings of both clean seeds and berries

TABLE III

SEEDLING PRODUCTION FROM JUNIPERUS VIRGINIANA SEEDS PLANTED IN SOIL IN FLATS KEPT IN A BOARD-COVERED FRAME

Planting date		Seeds		Berries	
		Per cent seedling production (4×100 seeds)		No. of seedlings (4×50 berries)	
		1950	1951	1950	1951
1949	July 5	44	—	121	—
	Aug. 4	51	—	106	—
	Sept. 7	42	—	56	—
	Oct. 6	32	—	26	—
	Nov. 4	13	20*	0	24
	Dec. 5	33	65	22	164
1950	Jan. 4	23	56	10	107
	Feb. 3	3	37	1	115
	Mar. 3	—	13**	—	107
	April 4	—	50	—	124
	May 4	—	49	—	114
	June 5	—	57	—	199

* 2×100 Seeds only.
** Chipmunks ate some of the seeds.

were made each month throughout a year in the soil mixture described above. Duplicate flats with duplicates of 100 seeds or 50 berries each were used. After planting, the flats were placed in a cold frame, protected by a board cover during the winter months.

Satisfactory seedling stands were secured the first spring after plantings made from April 4 to September 7 inclusive. Very few additional seedlings can be expected the second spring from such plantings. On the other hand, seeding as late as October 6 to March 3 resulted in limited seedling production the first spring, but additional seedlings appeared the second spring. These effects were predictable on the basis of the experimental results described above. When an insufficient amount of warm weather follows planting, the cold of the winter after-ripens only those seeds with permeable coats, usually a small percentage of the entire lot. Another

summer is then required for making the coats permeable before the winter temperatures can after-ripen the embryo. When, on the other hand, plantings are made to ensure four to eight weeks of warm temperature, which permits the soil micro-organisms to render all seed coats permeable, after-ripening of all seeds can be effected the following winter. It should not be overlooked that a winter temperature of at least 5° C. is necessary for breaking the dormancy of the embryos after the coats have been made permeable. Also, it should be emphasized that 1° C. is less effective than 5° C. If possible the ground in which the seeds are planted should not be allowed to freeze. If it is necessary to store the seeds for a considerable length of time before planting, they should be kept at low temperature, preferably below freezing. Some germination failures may be due to loss of viability before planting. Detailed data on the keeping quality of *Juniperus* seeds are not available.

SUMMARY

Seeds of *Juniperus virginiana* possess dormant embryos which require a period of three months at 5° C. to after-ripen. One degree C. is less effective, and 10° C. is totally ineffective. Failure to obtain complete germination after optimum low temperature treatment is due to the presence of impermeable coats in a large percentage of the seeds. Seed coats may be made permeable by exposure to moisture at a temperature of approximately 25° C. for two to eight weeks, or by soaking for 30 minutes in concentrated sulphuric acid. Seed coat treatment should be given before the seeds are placed at low temperature for breaking the dormancy of the embryos.

Spring or summer planting out-of-doors in regions which have low winter temperatures results in complete seedling production the following spring.

LITERATURE CITED

1. AFANASIEV, M., and M. CRESS. Producing seedlings of Eastern red cedar, *Juniperus virginiana* L. Oklahoma Agric. Exp. Sta. Bull. 256: 21 pp. 1942.
2. BARTON, LELA V. Experiments at Boyce Thompson Institute on germination and dormancy in seeds. Sci. Hort. **7**: 186–193. 1939.
3. CHADWICK, L. C. Seeds of red cedar. Amer. Nurseryman **83**(9): 10. May 1, 1946.
4. GERBRACHT, J. H. Evergreens from seed. North & South Dakota Hort. **10**: 46, 48. 1937.
5. JELLEY, M. E. Eastern red cedar. Jour. Forest. **35**: 865–867. 1937.
6. PACK, DEAN A. After-ripening and germination of *Juniperus* seeds. Bot. Gaz. **71**: 32–60. 1921.
7. PARKER, JOHNSON. Germination of eastern redcedar seeds. Jour. Forest. **48**: 255–256. 1950.
8. WEBSTER, C. B., and GEO. T. RATLIFFE. A method of forcing quick germination of *Juniperus virginiana* L. seeds. Jour. Forest. **40**: 268. 1942.

VIRUS DISEASES OF TOBACCO MOSAIC AND POTATO YELLOW DWARF NOT CONTROLLED BY CERTAIN PURIFIED ANTIBIOTICS

Helen Purdy Beale and Carolyn R. Jones

INTRODUCTION

Few claims can be made at present for any appreciable degree of success in the antibiotic therapy of diseases caused by the smaller or "true" viruses (1, 6, 12). Leben and Fulton (10) in a preliminary account have reported an inhibition in symptom expression of two plant viruses attributable to the presence of various chemicals, of which two are antibiotics, namely, streptothrycin hydrochloride and terramycin hydrochloride in a concentration of 1000 p.p.m. Six other antibiotics, antimycin A, bacitracin, chloramphenicol, neomycin, penicillin G (K salt) and streptomycin sulfate, were also tested by these investigators with negative results. Another report (4) states that streptomycin did not inhibit the development of typical symptoms of tobacco-mosaic disease in either Turkish tobacco, *Nicotiana tabacum* L., a Turkish variety, or *N. glutinosa* L. Earlier research on the subject of antibiosis in plant viruses has already been reviewed by the senior author (13) and will not be repeated in this paper except to note the publication of an article in full, available for review only in abstract form (Gupta and Price, 7).

The authors of the present article made tests for the control of two of the smaller viruses, those of tobacco mosaic and potato yellow dwarf with the purified antibiotics, penicillin, streptomycin, chloramphenicol (chloromycetin), aureomycin, and terramycin. The results of these tests already have been reported (13, p. 257) as negative. Tobacco-mosaic virus, one of the most stable plant viruses, was selected because it has been studied so widely that various techniques have been developed which facilitate its handling. Many independent and varying estimates of the length of this rod-shaped virus have been made, employing a number of different physical methods. The dimensions obtained most frequently by different investigators agree fairly well with those published in 1950 by Knight (9) and based upon an electron microscopic examination, whereby a width of 15 and a length of 300 millimicrons is reported. Potato yellow dwarf, a labile, leafhopper-borne virus, capable of mechanical transmission to Indian tobacco (*Nicotiana rustica* L.) with the aid of abrasives, was chosen because of its larger size. Measurements made on the particle size by Brakke, Black, and Wyckoff (3) indicate that it is sphere-like with a diameter of 110 millimicrons and has a sedimentation constant of 1130

Copyright, 1952, by Boyce Thompson Institute for Plant Research, Inc.

Svedberg units, which as Black (2, p. 220) states, " . . . probably is the largest plant virus studied in the ultracentrifuge up to the present time." The virus is readily filterable through Berkefeld filters, N. and W.

PRELIMINARY EXPERIMENTS

Before deciding upon the most effective method to adopt for testing the antibiotics for the control of these virus diseases, some preliminary tests were made.

Detached leaves of *N. glutinosa*, a local-lesion host of tobacco-mosaic virus, were placed in beakers with their petioles immersed in a solution of terramycin containing 100 p.p.m., or in tap water as a control. The leaves of different plants were equally distributed among four beakers and one set each of treated and untreated leaves was held in the dark and the corresponding sets, in the light. The leaves were inoculated with virus just prior to detachment from the plant, and no inhibition of virus multiplication occurred in any of the four sets.

A single futile attempt was made to cure a diseased plant after the virus had become established. The tip of a mosaic-diseased tobacco plant was excised and immersed in a solution of 250 to 500 p.p.m. aureomycin hydrochloride. The test was run for two weeks and the solution of antibiotic was changed two or three times daily but the virus content in the tip remained high.

Adding the antibiotic to plants potted in sand or soil, a method formerly used by the writers for other chemicals, was not tried because of the larger amount of solution required and the possible decomposition of the antibiotics in soil or their adsorption on the sand or soil particles.

As a result of these various preliminary experiments, the principal procedure adopted for testing the antibiotics for the control of the two virus diseases was the wick method. It was thought that if the antibiotic actually possessed the power to exert any inhibitory action on either virus, this procedure for treating a systemic host should be the most effective in controlling the disease.

THE WICK METHOD OF TREATING PLANTS WITH CHEMICALS

MATERIALS AND METHODS

Antibiotic solutions used. Purified sodium penicillin, streptomycin sulfate, and sodium terramycin were kindly supplied by Dr. G. L. Hobby of Chas. Pfizer & Co., Inc., aureomycin hydrochloride was provided through the courtesy of Lederle & Co., and a sample of chloramphenicol (chloromycetin) was obtained from Parke, Davis & Co. Some additional potassium penicillin G and chloramphenicol were purchased to complete the experiments. Aureomycin hydrochloride was usually dissolved in a 0.0267

M glycine buffer, while the other four antibiotics were used generally in a 0.0125 M potassium phosphate buffer at pH 6.9. In a single experiment *p*-aminobenzoic acid was tested with penicillin and streptomycin for possible synergistic action. In one test, Turkish tobacco was treated on successive days with repeated doses of streptomycin, followed by aureomycin and then penicillin since a combination of antibiotics may control an infection when one alone fails.

The concentration of each antibiotic to be used was determined chiefly by its toxicity to the plants. The highest concentration tolerated by the plant was generally employed.

Inoculation of plants with virus. Young plants of a Turkish variety of tobacco, *N. tabacum*, with a range of 2.1 to 18.5 cm. in height were used for the experiments with tobacco mosaic. Cheesecloth inoculation pads moistened with purified preparations of the virus in a concentration of about 0.2 mg. per ml. were rubbed lightly on a single leaf, either directly above, or below, or on a level with the place on the stem where the wick was threaded into the plant. If typical symptoms of tobacco-mosaic disease were lacking at the termination of an experiment, the tip and stem of the plant in question were macerated and inoculated on *N. glutinosa* to determine the presence or absence of virus. In a few cases, antibiotic therapy was continued for several days after the plants had succumbed to the disease in futile attempts to induce recovery.

In some tests, a "pure strain" of virus was used for inoculation. This single strain was isolated by the inoculation of the systemic host plant, Turkish tobacco, with a single local lesion induced by the virus on its local-lesion host, *N. glutinosa*. The alternate inoculation of the two hosts was repeated until it seemed likely that a single strain was obtained. With this source of inoculum, the virus was not purified but was used as the expressed sap from an infected Turkish tobacco plant. The object of isolating a pure strain for inoculation purposes in preference to using a mixture of strains, was to lessen the possibility of building up in the plant a high concentration of a strain resistant to the antibiotic at the expense of more susceptible ones.

N. rustica was used in the studies with potato yellow dwarf. The original virus was kindly supplied by Dr. L. M. Black. The procedure for testing an antibiotic for the possible control of the disease was similar to that described above for tobacco mosaic except that in order to obtain a high percentage of infection, it was necessary to inoculate several leaves on each plant so as to include the most susceptible age, and to sprinkle carborundum on the leaves to be inoculated, and finally to remove the growing tip of the inoculated plant and pinch off all the secondary shoots forming during the period of incubation. In doubtful cases of infection with the potato-yellow-dwarf virus, healthy *N. rustica* was inoculated with

macerated material from the leaves and stem of the plant showing obscure symptoms.

In addition to the experiments with the systemic host of tobacco-mosaic virus, Turkish tobacco, a series of supplementary tests was included with the local-lesion host, *N. glutinosa*. Each antibiotic solution was mixed separately *in vitro* with a given concentration of virus and then inoculated on opposite half-leaves or on whole leaves of *N. glutinosa* plants, according to the Latin-square arrangement. The results from the latter method were recorded and subjected to an analysis of variance to determine a possible significant difference in the various treatments.

Introduction of chemicals into plants. Solutions of the antibiotics were introduced into the plant by the "thread method" essentially as described by Roberts (11) and Felber (5) but with a few modifications which seemed to enhance the value of the technique. Instead of darning cotton which was tried at first, a glass fiber, purchased from the Atlas Asbestos Company of North Wales, Pennsylvania, under the trade name of "Plant Glaswik," was substituted. The glass wick was unraveled to a single strand and threaded into a darning needle which was passed through the center of the stem near the base at a ninety degree angle. Both ends of the wick protruding from either side of the shoot were dipped into a vial of antibiotic solution partially embedded in the soil near the shoot to prevent tipping over, as illustrated in Figure 1. The glass wick was preferred to cotton thread since it exerted strong capillary attraction and insured a continuous supply of the solution to the plant. Also, the glass fiber was not so likely as cotton to become contaminated with fungi in the humid greenhouses where the tests were conducted. The potted plants were set in saucers to permit watering from the bottom and the vials of antibiotic solution were further protected from splattering during watering by covering them with disks of wax paper, larger than the top of the pot, and slit down the radial line toward the center, where a small circle was cut out to accommodate the stem. These paper covers were held in place by two wires, stuck into the soil through the margin of the paper disk on opposite sides and hooked over the edge of the pot. The approximate amount of antibiotic absorbed by each plant was calculated from the volume required for replacement in the vials at various intervals during the experiment, usually daily, when the antibiotic solution was replaced with a fresh solution. In one experiment with aureomycin the solution was changed twice daily. No correction was made for evaporation which was slight due to the humid layer of air above the surface of the liquid in the vials created by the wax paper covers over the moist soil. In the case of the corresponding control plants in each test, distilled or tap water or a buffer solution was substituted for the antibiotic solution. Pretreatment of a plant with an antibiotic was usually given at least 24 hours before inoculation to allow distribution throughout

the plant before the virus was introduced. An experiment was terminated when all of the control plants became diseased, or within 9 to 25 days when 100 per cent infection of the controls was not obtained.

In order to determine the probable distribution of a chemical introduced into the vascular system of a plant by a wick, a solution of 2,3,5-

FIGURE 1. Method of introducing the antibiotic into Turkish tobacco plant. A glass wick has been threaded through the shoot and both ends dipped into the antibiotic solution in a glass vial and embedded in the soil.

triphenyltetrazolium chloride was selected for trial since the original colorless preparation turns pink upon reduction within the plant. After several small scale tests, an experiment was undertaken involving 20 Turkish tobacco plants with a mean height of 17.5 cm. and an average of 9 leaves, and 6 Indian tobacco plants having a mean height of 14.0 cm. and an average of 8 leaves. Within three to four hours after treatment was begun, the chemical had reached the tips of all the plants, as evidenced by the

TABLE I

TESTS FOR THE CONTROL OF TOBACCO-MOSAIC DISEASE IN TURKISH TOBACCO PLANTS WITH ANTIBIOTICS

Antibiotic*	Concentration		Period of treatment, days	Absorption per plant			No. plants, diseased/treated
				Mean total prior to inoculation, mg.	Daily mean		
					Diseased	Healthy	
	P.p.m.	Molar			Ml. (mg.)	Ml. (mg.)	
Penicillin**	300		14	6.3	5.3 (1.6)		5/5
Streptomycin**	1000, 500, 250		9–13	20.7	6.7 (4.3)		6/6
Distilled water							
Phosphate buffer, pH 6.8		.0125			3.3		4/4
Penicillin and PABA	250		12–14	3.0	2.7 (0.68)	2.5 (0.63)	5/8
Streptomycin and PABA	800		14–17	9.4	2.3 (1.8)	3.3 (2.6)	7/8
Distilled water					2.0	2.8	5/8
p-Aminobenzoic acid, pH 6.6–6.9		.0036			2.5		8/8
Streptomycin	130		17–25	1.9	1.9 (0.25)		6/6
Phosphate buffer, pH 6.8		.0125			2.7		6/6
Streptomycin in phosphate buffer, pH 7.0	500	14		1.2	2.6 (1.3)		8/8
Aureomycin in glycine buffer, pH 6.0–6.6	500			3.3	2.5 (1.3)		7/8
Phosphate buffer, pH 6.8		.0125			10.5		6/6
Glycine buffer, pH 6.4		.0067			10.4		6/6
Streptomycin**	2000, 1000, 500		10–14	6.2	3.1 (1.8)	2.6 (2.4)	4/6
Distilled water							
Phosphate buffer, pH 7.0		.0125			3.1		6/6

* The sodium salts of penicillin and terramycin, the sulfate of streptomycin, and the hydrochloride of aureomycin were used. Unless otherwise stated, the diluent for the anti-

TABLE I—*Continued*

Antibiotic*	Concentration		Period of treatment, days	Absorption per plant			No. plants, diseased / treated
				Mean total prior to inoculation, mg.	Daily mean		
					Diseased	Healthy	
	P.p.m.	Molar			Ml. (mg.)	Ml. (mg.)	
Aureomycin† in glycine buffer	1000, 500	.0267	8–9	2.1	2.8 (1.2)	3.7 (1.4)	5/6
Glycine buffer, pH 4.2		.02			2.7		6/6
Aureomycin†	500, 250	.0167	8–10	3.1	4.0 (1.7)		6/6
Glycine buffer, pH 7.5		.02			2.7		5/5
Aureomycin in phosphate buffer, pH 6.0–6.3	1000, 500	.01, .005		8.1	7.9 (3.9)		5/5
Aureomycin in glycine buffer, pH 6.4–6.8	1000, 500, 250	.0133, .0067	13	9.3	6.8 (3.4)		5/5
Terramycin in dilute HCl	200				3.1 (0.62)		3/3
Phosphate buffer, pH 6.0		.01, .005			5.2		6/6
Glycine buffer, pH 6.4		.0133, .0067, .0033			5.2		4/4
Tap water					6.1		5/5
Terramycin, dissolved in HCl, pH 7.0	250			6.2	7.9 (2.0)		8/8
Terramycin, dissolved in NaOH, pH 7.0	250			5.1	8.1 (2.0)		10/10
Distilled water					9.5		9/9
Terramycin	62.5	.01	14	1.1	8.0 (0.5)		20/20
Phosphate buffer, pH 6.8–7.0		.02			8.1	7.1	9/10

biotic was the buffer solution used for the control plants at the molar and H-ion concentrations indicated.

** Replaced with fresh antibiotic solution daily. Plants were inoculated with a pure strain of the virus.

† Replaced with fresh antibiotic solution twice daily. Plants were inoculated with pure strain of virus.

deep-pink colored leaves, and within 18 hours the dye had reached every part of the plant above soil level and had stained the tap root as well. Frequently, an initial alternate half-leaf absorption of the dye was observed, indicating that the wick had penetrated only a limited portion of the vascular system, but in less than 18 hours the one-sided absorption was obscured by an almost completely uniform distribution of the dye. When the wick was threaded into the petiole of a leaf, the dye reached the leaf directly above the treated one first. The rate of distribution of the liquid to all parts of the plant depended upon the extent to which the vascular system was penetrated by the wick and upon the rate of transpiration of the plant. The most rapid widespread distribution of the chemical was obtained by threading the wick into the shoot midway between two leaves 1.5 to 3.0 cm. from the soil level and equidistant from the strands of tissue extending down the stem from the two leaves on opposite sides.

RESULTS OF TREATMENTS WITH ANTIBIOTICS BY THE WICK METHOD

Turkish tobacco treated for the control of tobacco-mosaic disease. The results of these experiments are recorded in detail in Table I. In two separate tests in which it was calculated that a total of 13 plants absorbed an average of 4.6 mg. of penicillin before inoculation, 10 became infected as compared to 17 of 20 control plants. In five tests, in which 34 plants received an average of 7.9 mg. of streptomycin prior to inoculation, 31 plants succumbed to the disease while 35 of 38 control plants became infected. When *p*-aminobenzoic acid was tested as a synergist with both of these antibiotics, no significant inhibition of virus multiplication was obtained. Out of a total of 30 plants, which absorbed an average of 5.2 mg. of aureo-

TABLE II

TESTS FOR THE CONTROL OF TOBACCO-MOSAIC DISEASE IN TURKISH TOBACCO PLANTS WITH REPEATED DOSES OF STREPTOMYCIN, AUREOMYCIN, AND PENICILLIN SUCCESSIVELY

Antibiotic*	Diluent	Concn., p.p.m.	Period of treatment, days	Absorption per plant		No. plants treated, 100% diseased**
				Mean total prior to inoculation, mg.	Daily mean Ml. (mg.)	
Streptomycin	.0125 M phosphate buffer at pH 6.8	1000, 500	12–14	8.8	7.3 (5.7)	6
Aureomycin	.0267, .0133 M glycine buffer	1000, 500		7.4	7.2 (5.3)	
Penicillin	.0125 M phosphate buffer at pH 6.8	312.5		1.6	6.1 (1.8)	

* For salts of antibiotics used see Table I, footnote.*

** The plants were inoculated with a pure strain of the virus. For control plants see Table I, first test.

TABLE III

TESTS FOR THE CONTROL OF POTATO-YELLOW-DWARF DISEASE IN INDIAN TOBACCO PLANTS WITH ANTIBIOTICS

Antibiotic*	Concn., p.p.m.	pH	Period of treatment, days	Absorption per plant		No. plants treated, 100% diseased
				Mean total prior to inoculation, mg.	Daily mean Ml. (mg.)	
Penicillin G**	500 (units)	6.7	12	Not recorded. Plants inoculated 4 hours after wicking.	9.6 (4800 units)	6
Streptomycin	1000	6.7			12.0 (12.0)	6
Chloramphenicol in distilled water	1000	7.1			9.2 (9.2)	2 (4 plants died)
Aureomycin in distilled water	1000	5.6			5.1 (5.1)	6
Terramycin	200	6.8			9.2 (1.8)	6
.01 M phosphate buffer		6.7			7.8	6
Chloramphenicol in distilled water	166.5		14	4.5†	9.7 (1.6)	6
Terramycin in .01 M phosphate buffer	400	5.0			6.6 (2.6)	6
Tap water					5.8	6

* The potassium salt of penicillin G was used. For salts of other antibiotics and diluents employed see Table I, footnote *.

** Tablet also contained sodium carbonate, sodium benzoate and citric acid as buffer substances. Milligrams of penicillin not indicated, hence concentration is expressed in units.

† Average of four plants, the other two plants were inoculated on the day treatment began.

mycin before inoculation, 28 developed mosaic disease compared to 100 per cent of the 27 control plants. In the case of terramycin-treated plants, 38 plants absorbed an average of 4.1 mg. of the antibiotic prior to inoculation, and for an additional 3 plants the data were not available. The entire 41 plants became diseased, while 23 of 24 corresponding control plants developed mosaic.

When 6 plants were treated successively with repeated doses of streptomycin, aureomycin, and penicillin until 8.8 mg., 7.4 mg., and 1.6 mg. of antibiotic were absorbed respectively before inoculation, 100 per cent infection resulted, compared to 17 plants which became diseased out of 20 controls (see Table II).

Indian tobacco treated for the control of potato-yellow-dwarf disease. As recorded in Table III, 6 plants each were treated with penicillin, strepto-

TABLE IV

TESTS WITH ANTIBIOTICS FOR POSSIBLE INACTIVATION OF TOBACCO-MOSAIC VIRUS IN VITRO BY INOCULATION OF THE LOCAL-LESION HOST, NICOTIANA GLUTINOSA L.

Antibiotic*	Diluent	Antibiotic concn.		No. leaves inoculated with each inoculum**	Total No. lesions induced	No. lesions indicating difference† when	
		P.p.m.	pH			P = 5%	P = 1%
Aureomycin	.0267 M glycine buffer	1,000	6.3		1,348		
Penicillin	.0125 M phosphate buffer	2,000	6.9		1,574		
Streptomycin	.0125 M phosphate buffer	2,000	6.8		1,420		
Chloramphenicol, dissolved in ethanol	.0125 M phosphate buffer	2,000	6.9	18	1,142	528	699
Control	.0125 M phosphate buffer		6.9		1,019		
Control	.0133 M glycine buffer		6.2		1,582		
Aureomycin	.0167 M glycine buffer	250	6.4		1,313		
Control	.0133 M glycine buffer		5.8	72††	1,282		
Terramycin, dissolved in NaOH	Distilled water	886	7.0		2,087		
Terramycin, dissolved in HCl	Distilled water	843	7.0	24	2,292	725	959
Control	Distilled water				2,241‡		
Chloramphenicol, dissolved in ethanol	Distilled water	333		42	6,848	1,069	1,410
Control	Distilled water				6,400		
Terramycin	.01 M phosphate buffer	1,000	6.6		750		
Control	.01 M phosphate buffer			12††	677		
Terramycin	Distilled water	400	6.8	18	1,438	366	486
Control	Distilled water				1,679		

See footnotes on opposite page.

mycin, or aureomycin, and 12 plants each with chloramphenicol or terramycin. Although the average daily absorption of the antibiotic solution was considerable all of the 50 plants of the 54 treated, which were living at the termination of the experiments, became diseased as well as the 12 control plants.

RESULTS OF TESTING THE ANTIBIOTICS FOR A POSSIBLE INACTIVATION IN VITRO OF TOBACCO-MOSAIC VIRUS

In Table IV, the results of 6 separate tests in which penicillin, streptomycin, chloramphenicol, aureomycin, or terramycin were mixed with virus and then inoculated on the local-lesion host, *N. glutinosa*, failed to show a significant reduction in the number of lesions developing when compared with the control inocula of buffer or distilled water and virus.

DISCUSSION

Although no assay was made to determine the presence or the concentration of the various antibiotics in different parts of a treated plant, there are a number of indications that they were taken into the plant. In every case, the concentration of antibiotic used had to be adjusted to overcome the toxic effect not exerted by the diluent alone. Also, a cumulative toxic effect was observed which often resulted in the death of the plant. Frequently, the plants absorbed the entire contents of the vial leaving no indication of a residue of antibiotic. In the case of streptomycin (4), it has been shown that this antibiotic is absorbed through the roots of Turkish tobacco and may accumulate in the leaves and reach a concentration there approximately ten times as strong as that in the solution absorbed. As additional evidence of the absorption of streptomycin in the experiments described in the present article, lack of chlorophyll formation, a typical reaction to this antibiotic, was noted along the midribs and interveinal leaf tissues, and at times throughout the entire tips of the treated plants. Golden aureomycin was often evident in the orange-stained leaves of treated plants. The suggestion may be made that the plants so alter the absorbed antibiotic that its virus-inactivating power is destroyed. Since by definition, an antibiotic should withstand the action of enzymes and body fluids in order to qualify for successful therapy, and

---

* For salts of antibiotics used see Table I, footnote *.

** The antibiotic or buffer solution was mixed with an equal volume of tobacco virus, having a concentration of 0.2 mg./ml. The final concentration of both antibiotic and virus in the inoculum was thus reduced one-half.

† These numbers were obtained by an analysis of variance of data from leaves inoculated according to the Latin-square arrangement.

†† The half-leaf was used as the unit, instead of the whole leaf and the two inocula were tested on opposite half-leaves.

‡ Mean total number of lesions induced by three separate inocula on 24 leaves.

since the antibiotics used in these experiments have fulfilled the requirements for internal use in animals, they therefore possess a certain degree of proven stability.

It is of some interest to consider whether the treated plants received a dosage of antibiotic equivalent to that recommended for therapy in man. The necessary calculation was made for a single experiment with aureomycin and it was found that the amount of antibiotic absorbed usually exceeded that recommended for man.

Since during antibiotic therapy strains of microorganisms resistant to the particular antibiotic used often develop, pure strains of the tobacco virus as well as mixed strains were used for the inoculation of plants. The possible appearance of variants in the plants after inoculation with the pure strain could not be controlled but the number of different variants resulting would be markedly reduced, thereby minimizing the chances of the appearance of a resistant strain.

The fact that the antibiotics tested were ineffective in controlling the virus diseases of tobacco mosaic and potato yellow dwarf was indicated by the many similar unsuccessful attempts of others to control animal diseases due to the smaller viruses. To quote F. L. Horsfall, Jr. (8, p. 18), there is "a striking correlation between the particle size and the structural complexity of viruses and the effectiveness of available chemotherapeutic substances." Among the microorganisms found susceptible to these antibiotics, bacteria predominate. A number of the Rickettsiae are also sensitive to treatment with several of them, and continuing down the taxonomic scale, members of the psittacosis-granuloma group, which occupy a place intermediate between the Rickettsiae and the smaller or true viruses, are also affected. Some of these antibiotics are beneficial in the treatment of a still smaller etiologic agent, the virus of atypical pneumonia, which is reported to be larger than the true viruses and smaller than those of the psittacosis-granuloma group. The fact that a large group of diseases caused by small viruses does not respond to the kind of antibiotic therapy thus far made available cannot be adequately explained until more is known about the nature of antibiotic reactions.

SUMMARY AND CONCLUSIONS

1. Solutions of the purified antibiotics, sodium penicillin, streptomycin sulfate, chloramphenicol, aureomycin hydrochloride, or sodium terramycin were introduced by means of a wick into Turkish tobacco (*Nicotiana tabacum* L.) or Indian tobacco (*Nicotiana rustica* L.) and the plants were inoculated later with tobacco-mosaic virus and the virus of potato yellow dwarf, respectively.

2. In a single experiment, streptomycin, aureomycin, and penicillin

were introduced into Turkish tobacco successively and in repeated doses. The plants were inoculated later with tobacco-mosaic virus.

3. The same antibiotics were tested *in vitro* for the possible inactivation of tobacco-mosaic virus. The antibiotic-virus mixtures were assayed on the local-lesion host, *Nicotiana glutinosa* L.

4. It was concluded that under the conditions of the experiment, all of the antibiotics tested were ineffective in controlling the virus diseases of tobacco mosaic or potato yellow dwarf.

LITERATURE CITED

1. BARON, A. L. Handbook of antibiotics. 303 pp. Reinhold Publishing Corp., New York, N. Y. 1950.
2. BLACK, L. M. Further studies on the properties of potato yellow-dwarf virus. Phytopath. **41**: 213–220. 1951.
3. BRAKKE, M. K., L. M. BLACK, and R. W. G. WYCKOFF. Correlation of potato yellow-dwarf virus bodies with infectivity during centrifugation. Phytopath. **40**: 3. 1950.
4. Common tobacco-mosaic virus not affected by streptomycin. Illinois Agric. Exp. Sta. Rept. **1947/48**: 178. 1950.
5. FELBER, IRMA M. Direct introduction of chemical substances in herbaceous plants. Science **106**: 251. 1947.
6. FLOREY, H. W., and others. Antibiotics. Vols. 1, 2. 1774 pp. Oxford University Press, London, New York, Toronto. 1949.
7. GUPTA, B. M., and W. C. PRICE. Production of plant virus inhibitors by fungi. Phytopath. **40**: 642–652. 1950.
8. HORSFALL, FRANK L., JR. Advances in the diagnosis of virus infections. Bull. New York Acad. Med. **26**(1): 3–19. 1950.
9. KNIGHT, C. A. Some salient points concerning plant viruses. *In* Delbrück, M., editor. Viruses. pp. 52–54. California Inst. Tech., Pasadena, Calif. 1950.
10. LEBEN, CURT, and R. W. FULTON. The inhibition of virus symptom expression by sodium azide, potassium cyanide, and two antibiotics. Phytopath. **41**: 23. 1951.
11. ROBERTS, WINIFRED O. Simplifications of the Roach method of diagnostic plant injection. Jour. Pomol. & Hort. Sci. **22**(3/4): 184–188. 1946.
12. Terramycin. Ann. New York Acad. Sci. **53**, Art. 2: 223–460. 1950.
13. WEINDLING, R., H. KATZNELSON, and HELEN PURDY BEALE. Antibiosis in relation to plant diseases. Ann. Rev. Microbiol. **4**: 247–260. 1950.

ACTIVATION OF SODIUM 2-(2,4-DICHLOROPHENOXY)-ETHYL SULFATE

ROBERT B. CARROLL

Thousands of tons of 2,4-D have been used to control weeds during the nine years since Zimmerman and Hitchcock discovered its growth-regulating properties. The application of 2,4-D by power sprayers and airplanes has resulted in serious drift damage in some localities to many sensitive crops such as tomatoes and cotton. This has prompted considerable discussion by legislative assemblies and in some states led to the establishment of laws requiring the operators of spraying equipment to be bonded.

The need for a safer material that would not injure nearby sensitive crops was met by King and Lambrech (6) in 1949 when they found that sodium 2-(2,4-dichlorophenoxy)ethyl sulfate (CRAG Herbicide 1, E. H. 1) was an excellent compound for use under conditions where drift hazard with 2,4-D was high and where the weed population was in the early stages of germination. This material is relatively non-toxic when applied to foliage of cotton, tomato and other plants sensitive to 2,4-D but limits the growth of young weed seedlings when used in pre- or post-emergence applications (3). King, Lambrech, and Finn (7) believe that the material is activated by some soil factor, presumably biotic in nature, since it becomes more active in unsterilized soil than in soil which has been steam sterilized.

Results of tests on the effect of hydrogen-ion concentration on the activation of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate are reported in the present paper. Low pH values resulted in hydrolysis of the compound with the formation of 2,4-dichlorophenoxyethanol, which was biologically active. Tests were also carried out on the effect of riboflavin in counteracting the activity of sodium 2,4-D and related compounds.

MATERIALS AND METHODS

The activity of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate and related compounds such as 2,4-D and the corresponding ethanol compound was measured in this investigation by the suppression of primary root elongation of cucumber (*Cucumis sativus* L. variety Early Fortune) seedlings using a method similar to that described by Ready and Grant (9). In some of the tests to be described, germination and growth of the test seedlings were carried out in light. Light was furnished by means of two parallel banks of 48-inch, 40-watt, daylight fluorescent tube lights mounted approximately one foot above the table on which the Petri dishes containing the seed were distributed.

Copyright, 1952, by Boyce Thompson Institute for Plant Research, Inc.

The responses of young tomato (*Lycopersicon esculentum* Mill. variety Bonny Best) plants to the chemicals were measured by four different techniques, the first three of which, involving leaf immersion, excised tomato shoots, and soil additions, have been described by King (5). The fourth method was that of total plant immersion described by Mullison (8) as a rapid evaluation test. All the tomato plants were grown in Merrimac loam (11) composted for three years prior to use with equal parts of manure and leaf mold. The plants were maintained under average greenhouse conditions and were watered twice each day as uniformly as possible. Special care was exercised in watering to prevent the test chemicals from being washed off the foliage and into the soil.

The effect of hydrogen-ion concentration on the activation of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate was determined in a buffered nutrient medium described by Tarr and Noble (10). The hydrogen-ion concentration was adjusted by titration to the desired level (pH 3.0 to 8.0) with 0.1 N NaOH or HCl using a Beckman Model G pH-meter. Sodium 2-(2,4-dichlorophenoxy)ethyl sulfate was added to some flasks at a concentration of 0.01 per cent and the medium steam sterilized at 245° F. for 30 minutes. Solutions containing sodium 2-(2,4-dichlorophenoxy)ethyl sulfate and their controls at pH 3.0 to 8.0 were used to moisten filter pads for duplicate test plates in the cucumber root suppression test.

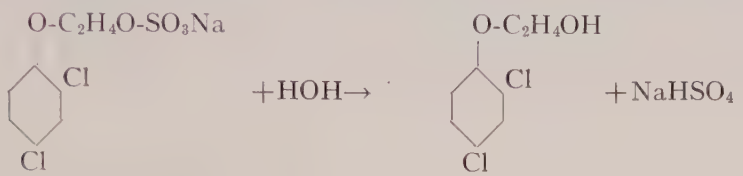
EXPERIMENTAL RESULTS AND DISCUSSION

CUCUMBER ROOT ELONGATION

In order to eliminate the possibility of any physical effect of soil on the activation of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate, preliminary experiments were performed using freshly made filtered soil extracts. When these data were compared with those obtained with solutions in tap or distilled water, it was evident that the soil extract was very much more effective in activating unsterilized solutions of the chemical. Further, a comparison made at the same time with several samples of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate prepared by different procedures indicated that impurities tended to mask activation. All further tests to be described were therefore performed using a sample of high purity.

Relation of 2,4-dichlorophenoxyethanol to growth inhibition. During preliminary investigations crystal formation was observed when slightly acid (pH 5.6) solutions of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate which had been sterilized in distilled water, were allowed to stand overnight. These crystals when filtered and dried and studied spectrographically with infra-red light proved to be those of 2,4-dichlorophenoxyethanol mixed with crystals of some inorganic compound. Samples of the solution were withdrawn and tested for sulfate ion by means of cold barium chloride. These gave positive tests for the sulfate ion almost instantaneously following acidulation.

When sodium 2-(2,4-dichlorophenoxy)ethyl sulfate undergoes hydrolysis in acid solutions sodium acid sulfate and 2,4-dichlorophenoxyethanol are formed as shown below:



Sodium 2-(2,4-dichlorophenoxy)ethyl sulfate 2,4-Dichlorophenoxyethanol

A sample of 2,4-dichlorophenoxyethanol purified by recrystallization was used to prepare a series of solutions containing from 0.01 p.p.m. to

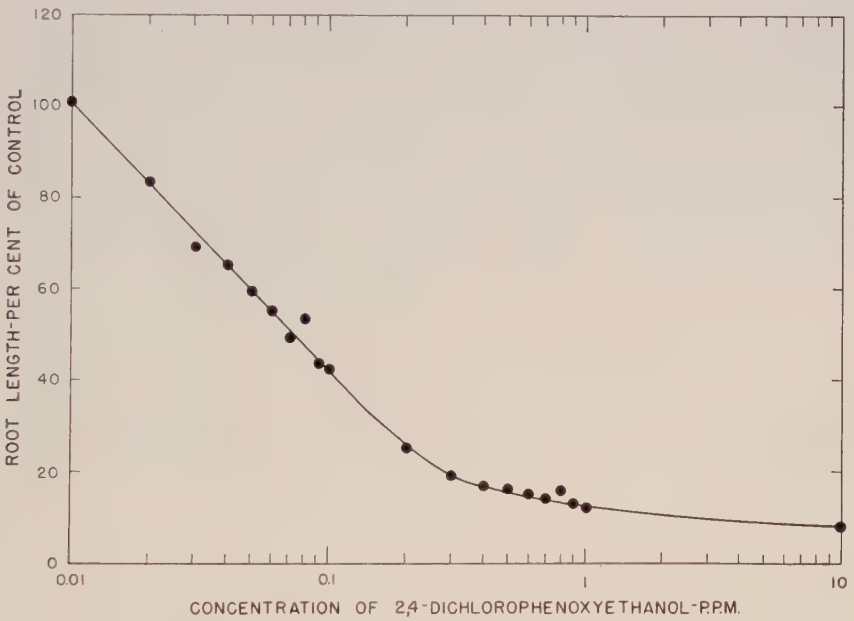


FIGURE 1. Standard curve of the inhibition of cucumber root elongation by 2,4-dichlorophenoxyethanol.

10.0 p.p.m. of the chemical. These solutions were used in the cucumber bioassay to determine the amount of inhibition produced by this chemical. These data are portrayed in Figure 1. Very low concentrations were effective in producing a marked inhibition of cucumber root elongation.

Effect of sterilization on growth inhibition. When the effect of pH on activity of solutions of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate was investigated more thoroughly, hydrogen-ion concentration was found to have little effect on activity unless a pH level of 4 or less was employed. Further, when comparisons were made between the activities of sterilized

and unsterilized buffered nutrient solutions containing 0.01 per cent of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate, sterilization was demonstrated to have little effect on activity at pH levels of 4 or above. Below a pH 4 sterilization appeared to increase the activity of the solutions (see Fig. 2). The activity of these solutions at pH levels of 5 to 8 corresponds to approximately 0.07 p.p.m. of 2,4-dichlorophenoxyethanol.

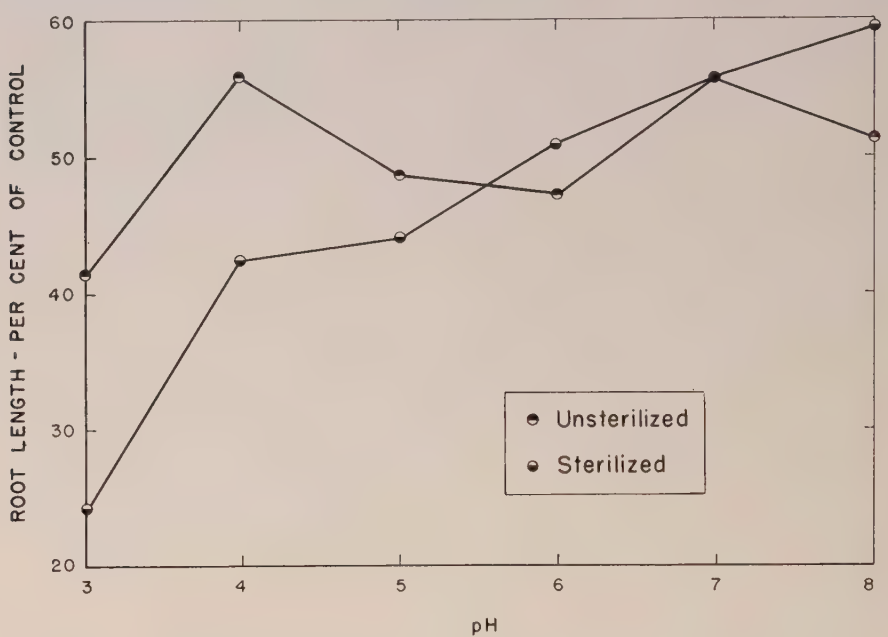


FIGURE 2. The effect of sterilization on the inhibition of root elongation produced in cucumber seedlings by a 0.01 per cent solution of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate, at six pH levels.

The reason for the approximately 40 per cent inhibition of root elongation of the cucumber seedlings receiving neutral or slightly alkaline solutions of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate is not known. The sample might have contained the small amount of 2,4-dichlorophenoxyethanol necessary to produce this inhibition or some buffer interaction may have obscured the response.

SOIL TESTS

The results reported by King *et al.* (7) were confirmed for greenhouse plantings in that the response of corn, cotton, soybeans, and tomato to sodium 2-(2,4-dichlorophenoxy)ethyl sulfate was greater in unsterilized soil than in soil which had been autoclaved immediately before use.

A series of comparisons was made between sodium 2-(2,4-dichlorophenoxy)ethyl sulfate, 2,4-dichlorophenoxyethanol and sodium 2,4-D by leaf

FIGURE 3. Tomato plants treated with solutions of (A) sodium 2,4-D; (B) 2,4-dichlorophenoxyethanol; and (C) sodium 2-(2,4-dichlorophenoxy)ethyl sulfate by soil applications. Left to right: water control, 100, 10, 1, and 0.1 p.p.m. of chemical applied in 40 ml. of distilled water three days prior to photographing.

immersion, total plant immersion, soil applications, and solution applications to excised tomato plants. In general, sodium 2,4-D was found to be more active than the ethanol compound which, in turn, was more active than sodium 2-(2,4-dichlorophenoxy)ethyl sulfate. There was relatively little response following applications to foliage of either the ethanol compound or sodium 2-(2,4-dichlorophenoxy)ethyl sulfate. The response was more severe when solutions of either chemical were applied to the excised stem or to the roots of tomato plants. Comparisons of the response of tomato plants to soil applications of these chemicals are shown in Figure 3.

Why these chemicals should act when applied so that they might enter

the stem or roots and fail to do so when applied to foliage is not known. It may be that translocation of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate and 2,4-dichlorophenoxyethanol does not take place from the leaves through the phloem while it does move rapidly upward through the xylem, since when the tips of the leaves of tomato plants were removed and these immersed in a solution of 2,4-dichlorophenoxyethanol, the response was more pronounced than when the foliage was immersed in a solution of this chemical without excision of the leaf tip.

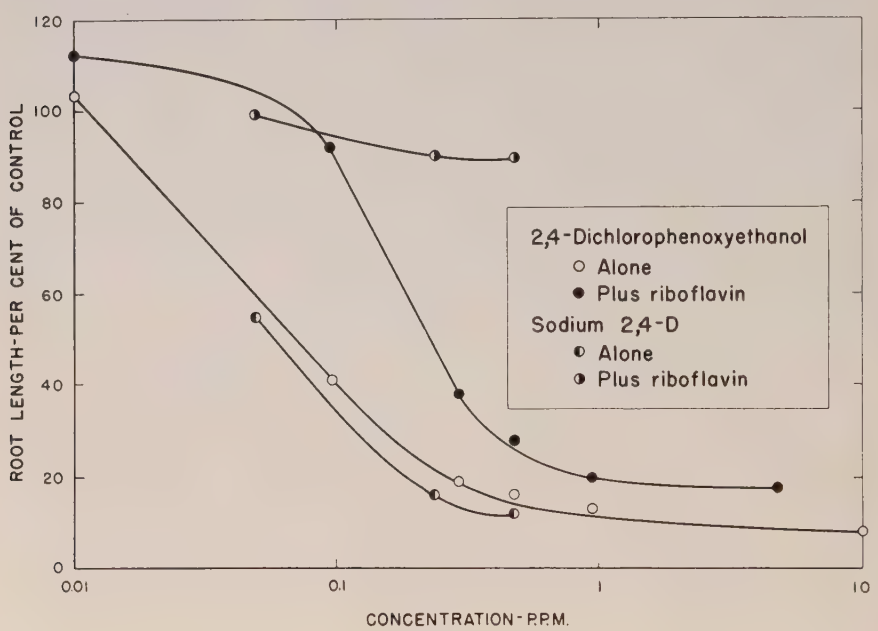


FIGURE 4. Percentage inhibition of cucumber seedlings under fluorescent lights when exposed to solutions of sodium 2,4-D and 2,4-dichlorophenoxyethanol alone and in combination with 50 p.p.m. of riboflavin.

THE USE OF RIBOFLAVIN TO COUNTERACT CHEMICAL EFFECTS

Previous studies with corn seedlings had shown that inhibition of corn root elongation by sodium 2,4-D could be counteracted by simultaneous exposure of the seedlings to solutions of riboflavin in light (1, 2). Using the cucumber bioassay, a comparison was made between the effect of sodium 2,4-D and 2,4-dichlorophenoxyethanol used alone and in combination with solutions of riboflavin. In both treatments the tests were carried out under fluorescent lights. These data on root response are presented in Figure 4. It is evident that the inhibition curves of both chemicals used alone are

approximately coincident over the range of concentrations used. It is also evident that riboflavin is much more effective in counteracting the inhibition produced by sodium 2,4-D than it is in counteracting the inhibition of 2,4-dichlorophenoxyethanol. This is further demonstrated when solutions containing 0.07 and 0.7 p.p.m. of the ethanol compound are combined with increasing amounts of riboflavin. The inhibition produced by the lower amount of the ethanol compound is effectively counteracted by use of 500 p.p.m. of riboflavin. At this same concentration of riboflavin maximum

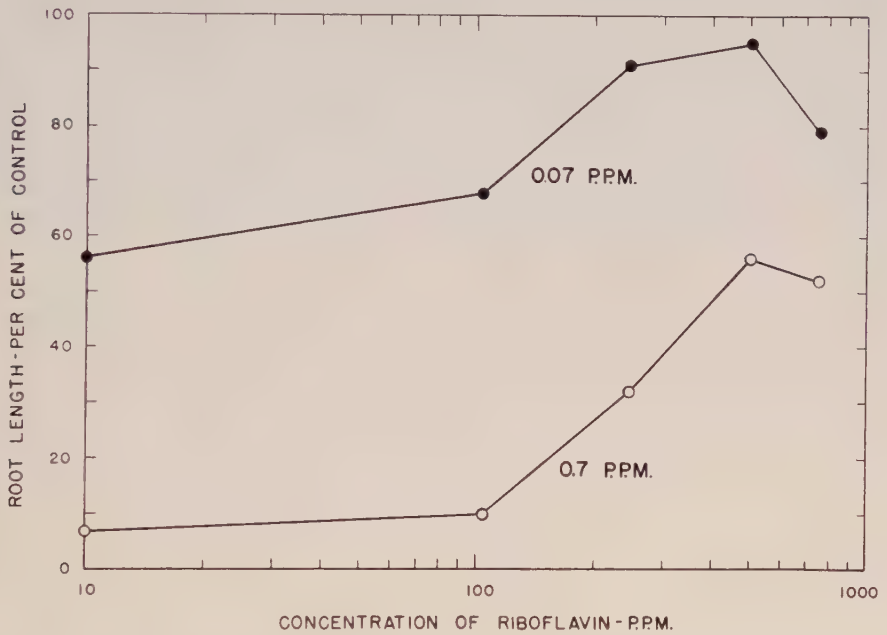


FIGURE 5. Inhibition of cucumber root elongation by 0.07 and 0.7 p.p.m. of 2,4-dichlorophenoxyethanol combined with various concentrations of riboflavin. Exposure to chemicals was carried out under fluorescent lights.

reduction of inhibition occurs when the higher concentration of the ethanol compound is used. However, inhibition is still pronounced (Fig. 5).

Riboflavin has been demonstrated by Galston (4) to be effective as a hydrogen carrier in the photo-oxidation of many indole-containing chemicals such as indoleacetic acid, tryptophane and others. In the presence of light, riboflavin becomes activated and can accept hydrogen from a suitable substrate. The reduced riboflavin is capable of transferring this hydrogen to oxygen directly, thus returning the riboflavin to its oxidized form. Riboflavin can also effect the photo-inactivation of at least three enzymes. Whether the present effect of riboflavin is due to either or both of these

phenomena is not now known. However, in view of the depression of the inhibition curve at the highest concentration of riboflavin used in the experiment shown in Figure 4, it seems probable that at this concentration, at least, riboflavin serves as an inhibitor of growth.

SUMMARY

A study of the effect of hydrogen-ion concentration and sterilization on the activity of solutions of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate revealed that hydrogen-ion concentration had little effect on activity above pH 4 while below this level activation increased with an increase in hydrogen-ion concentration. Further, steam sterilization increased the amount of activation at the lower pH levels.

Sodium 2-(2,4-dichlorophenoxy)ethyl sulfate on acid hydrolysis yielded 2,4-dichlorophenoxyethanol, a compound of high biological activity. Presumably, the other product of hydrolysis is sodium bisulfate. The chemical, 2,4-dichlorophenoxyethanol, was shown to have a high order of activity where inhibition of cucumber root elongation was used as a measure of activity. The chemical was only slightly active in producing epinastic responses in tomato plants when foliar applications of the chemical were made. The magnitude of activity was approximately 1/100th that of sodium 2,4-D. When solutions were applied to excised tomato stems or to the soil of potted tomato plants, the responses of tomato plants to this chemical were considerably more marked than when the tomato plants received foliar applications of the chemical.

It is suggested that the 2,4-dichlorophenoxyethanol does not travel as rapidly downward in the phloem with the photosynthate as does sodium 2,4-D. It appears to travel upward in the xylem at a much greater rate.

Riboflavin counteracts the inhibition produced by low dosages of 2,4-dichlorophenoxyethanol much less efficiently than it does similar dosages of sodium 2,4-D. The effect of 2,4-dichlorophenoxyethanol may either be more complex than that of sodium 2,4-D or be more permanent in its action.

LITERATURE CITED

1. CARROLL, ROBERT B. Apparent inactivation of 2,4-D by riboflavin in light. Amer. Jour. Bot. **36**: 821. 1949.
2. ———— Effectiveness of various chemicals in counteracting 2,4-D toxicity to corn seedlings. Thesis (Ph.D.) Louisiana State Univ. 88 pp. 1951.
3. FINN, THOMAS P., and L. J. KING. Weed control studies with Experimental Herbicide 1 at Seabrook Farms, Bridgeton, N. J. Proc. Northeastern Weed Control Conf. New York City, 1951. pp. 81–86. 1951.
4. GALSTON, ARTHUR W. Riboflavin-sensitized photoöxidation of indoleacetic acid and related compounds. Proc. Nat. Acad. Sci. **35**: 10–17. 1949.
5. KING, LAWRENCE J. A leaf immersion technique for studying the absorption and translocation of chemicals in plants. Contrib. Boyce Thompson Inst. **15**: 165–171. 1948.

6. KING, L. J., and J. A. LAMBRECH. A new chemical for use in pre-emergence weeding. Proc. Northeastern Weed Control Conf., New York City, **1949**: 34–36. 1949.
7. KING, LAWRENCE J., J. A. LAMBRECH, and THOMAS P. FINN. Herbicidal properties of sodium 2,4-dichlorophenoxyethyl sulfate. Contrib. Boyce Thompson Inst. **16**: 191–208. 1950.
8. MULLISON, WENDELL R. The tomato as a test plant for growth-regulators. Bot. Gaz. **112**: 521–524. 1951.
9. READY, DANIEL, and VIRGINIA Q. GRANT. A rapid sensitive method for determination of low concentrations of 2,4-dichlorophenoxyacetic acid in aqueous solution. Bot. Gaz. **109**: 39–44. 1947.
10. TARR, L. W., and S. C. NOBLE. The effect of hydrogen ion concentration upon the growth of seedlings. Delaware Agric. Exp. Sta. Bull. 131: 1–52. 1922.
11. VAN DUYNE, CORNELIUS, and J. H. BROMLEY. Soil survey of the White Plains area, New York. U. S. Dept. of Agric. Bur. of Soils. Field Operations of the Bureau of Soils, 1919. **21**: 563–606. 1925.

SUBSTITUTED BENZOIC ACIDS AS GROWTH REGULATORS

P. W. Zimmerman, A. E. Hitchcock, and Edward A. Prill

In recent publications (1, 12) 2,3,6-trichlorobenzoic acid and the corresponding aldehyde were shown to induce hormone-like responses in plants. These results are of special interest since they brought out the fact that derivatives of benzoic acid have physiological activity equal to that of well recognized growth regulants. Since the first report on the activity of substituted phenoxy and benzoic acids (11) appeared several other investigators also have published on this subject (2, 7, 10). The present report is concerned principally with three physiologically-active substituted benzoic acid derivatives. These are 2,6-dichlorobenzaldehyde, 2,6-dichlorobenzoic acid, and 2,6-dichloro-3-nitrobenzoic acid. These and several other substituted benzoic acids previously reported are compared.

PREPARATION OF COMPOUNDS

2,6-Dichlorobenzaldehyde. The Eastman practical grade of this compound was purified by steam distillation and recrystallization from petroleum ether to yield the pure compound melting at 70° C. (3, p. 226).

2,6-Dichlorobenzoic acid. The above purified aldehyde was oxidized to this acid by means of potassium permanganate. After isolation and recrystallization from carbon tetrachloride, the acid had a melting point of 143° to 144° C. which agrees with the highest reported values (3, p. 378).

2,6-Dichloro-3-nitrobenzoic acid. This acid was prepared by the nitration (5) of the above pure 2,6-dichlorobenzoic acid. After recrystallization from carbon tetrachloride it showed the reported melting point of 152° C.

3-Amino-2,6-dichlorobenzoic acid. The above nitro compound in ethanol solution was reduced with iron powder and hydrochloric acid. After recrystallization from water the new compound melted at 189° C. *Anal.* Calcd. for $C_7H_5O_2NCl_2$: Cl, 34.4. Found: Cl, 34.7.

2,3,4,5-Tetrachlorobenzoic acid (8), 2-chloro-6-nitrobenzoic acid (6), and 4,5-dichloro-2-nitrobenzoic acid (9). These compounds were synthesized according to methods given in the literature. The other compounds used were purchased or had been prepared in these laboratories at some previous time.

EXPERIMENTAL METHODS

Tomato (*Lycopersicon esculentum* Mill.) and stevia (*Piqueria trinervia* Cav.) were used as the principal test plants. Solutions of the three substances were applied to aerial parts and to soil in which the plants were growing. The aerial parts of the plants were treated with lanolin prepara-

Copyright, 1952, by Boyce Thompson Institute for Plant Research, Inc.

tions having a concentration range from 0.001 to 100 mg. per gram of lanolin. The chemical solutions were sprayed on the leaves by means of an atomizer. Water solutions containing 1 to 100 mg. in 30 to 50 ml. of water were applied to 500 grams of soil in which the plants were growing. Each treatment served as a test for one or more responses such as epinasty of leaves, curvatures, and inhibition of growth. The insoluble or only slightly soluble acids were formulated with triethanolamine.

The axillary angle of the leaf and stem was measured in degrees with a protractor or the plants were observed and ranked for relative change after treatment.

RESULTS

The responses induced for any given compound were similar regardless of the method of treatment. They are listed as cell elongation (associated with epinasty of leaves and curvatures of the stem), proliferation of cells (associated with induced cell division), induction of roots, modification of leaves and fruit, and inhibition of growth. In most respects the responses induced by 2,6-dichlorobenzoic acid and 2,6-dichloro-3-nitrobenzoic acid were similar to those reported for 2,3,6-trichlorobenzoic acid (12).

The comparative activity of the three compounds and threshold concentrations are shown in Table I. Table II shows the comparative activity of 2,6-dichlorobenzoic and 2,6-dichloro-3-nitrobenzoic acids when the plants were treated with four different concentrations of the chemicals in lanolin and ranked for degree of response. Table III shows the relative epinastic response when the chemical was applied to the soil. Table IV shows length of time required for the plant to respond to 0.5 per cent spray solutions of two benzoic acids.

TABLE I

EFFECTIVENESS OF THREE BENZOIC ACID DERIVATIVES FOR INDUCING VARIOUS RESPONSES WHEN APPLIED AS LANOLIN PREPARATIONS TO TOMATO PLANTS

Compound	Minimum effective concentration (mg./g.)			
	Cell elongation	Leaf modification	Root induction	Inhibition of growth or lethal effects
2,6-Dichlorobenzaldehyde	Inactive	5	Inactive	100
2,6-Dichlorobenzoic acid	10	0.5	25	25–50
2,6-Dichloro-3-nitrobenzoic acid	1	0.5	10	25–50

Regardless of how 2,6-dichlorobenzaldehyde was formulated and applied to plants, it failed to induce any of the responses involving cell elongation or cell division (epinasty of leaves, curvature, and induction of roots). It caused modification of leaves and inhibited growth. A 2 per cent

TABLE II

COMPARATIVE ACTIVITY OF 2,6-DICHLOROBENZOIC AND 2,6-DICHLORO-3-NITROBENZOIC ACIDS WHEN APPLIED AS LANOLIN PREPARATIONS TO TOMATO PLANTS

Compound	Concentration, mg./g.	Ranks for relative degree of response	
		Epinasty	Leaf modification
2,6-Dichloro-3-nitrobenzoic acid	100	1	1
2,6-Dichloro-3-nitrobenzoic acid	50	2	2
2,6-Dichloro-3-nitrobenzoic acid	25	3	3
2,6-Dichlorobenzoic acid	100	4	4
2,6-Dichloro-3-nitrobenzoic acid	10	5	5
2,6-Dichlorobenzoic acid	50	6	6
2,6-Dichlorobenzoic acid	25	7	7
2,6-Dichlorobenzoic acid	10	8	8*

* No response.

TABLE III

EPINASTIC RESPONSE OF TOMATO LEAVES TO 2,6-DICHLOROBENZOIC AND 2,6-DICHLORO-3-NITROBENZOIC ACIDS APPLIED TO THE SOIL

Compound	Treatment, mg. per 500 g. of soil	Relative epinastic response	
		Observed* after 6 hr.	Measured** after 48 hr.
Control	None	0	57°
2,6-Dichlorobenzoic acid	1	0	62°
	5	0	75°
	10	0	96°
	50	0	109°
2,6-Dichloro-3-nitrobenzoic acid	1	+	104°
	5	++	114°
	10	+++	122°
	50	++++	136°

* 0=No change; +=slight epinasty; ++=medium epinasty; +++=considerable epinasty; ++++=pronounced epinasty.

** Average of six leaves.

TABLE IV

COMPARATIVE ACTIVITY OF TWO BENZOIC ACID DERIVATIVES AND TIME REQUIRED TO INDUCE EPINASTY OF TOMATO LEAVES AFTER BEING SPRAYED WITH A 0.5 PER CENT SOLUTION

Compound	Average of twelve angles in degrees at six different times, hours					
	Original angle	1	2	3	6	24
Control	54	61	61	57	55	55
2,6-Dichlorobenzoic acid	65	68	72	70	76	94
2,6-Dichloro-3-nitrobenzoic acid	58	67	74	78	88	113

solution was toxic enought to kill plants within ten days after treatment. The only growth regulation of the aldehyde which resembled a hormone-like response was its capacity to modify the pattern of new organs. These results are in contrast with those obtained with 2,3,6-trichlorobenzaldehyde which was as active as the corresponding acid for cell elongation, induction of roots, and modification of organs.

2,6-Dichlorobenzoic acid was not as active as 2,6-dichloro-3-nitrobenzoic acid. The latter, however, was not as active as 2,3,6-trichlorobenzoic acid.

The toxicity of 2,6-dichlorobenzoic acid was higher than that of 2,6-dichloro-3-nitrobenzoic acid, but both compounds were so effective for inhibiting growth and eventual killing of the plants that they might be useful as herbicides. Three species of weeds, *Stellaria media* (L.) Cyrill, *Galinsoga parviflora* Cav., and *Polygonum* sp., growing with tomato plants and sprayed with 0.1 per cent of 2,6-dichlorobenzoic acid and 2,6-dichloro-3-nitrobenzoic acid were inhibited or killed. Where inhibited, the plants produced swollen stems and modified leaves as they resumed growth. A .1 per cent solution sprayed on tomato plants almost stopped elongation, and the plants continued to develop abnormal growths for 10 to 20 days.

The two derivatives were lethal to tomato plants when applied by means of an atomizer at a concentration of 1 per cent or more. A concentration of 0.001 per cent of 2,6-dichloro-3-nitrobenzoic acid induced epinasty while 0.5 per cent of 2,6-dichlorobenzoic acid was required for this response.

Table V shows a list of substituted benzoic acids and their comparative physiological activity for different types of responses. It will be seen from the results that 2-bromo-3-nitrobenzoic, 2,3,6-trichlorobenzoic, and 2,6-dichloro-3-nitrobenzoic acids are among the more active for cell elongation. Many of them are equally active for formative effects, and some of them show considerable toxicity and might make very effective herbicides.

Tests involving translocation of growth regulators were made with stevia plants. The movement of the substances in the tissue could be followed by the responses induced. The principal responses were modification of leaves, swelling of the nodes, and killing of tissue. When either water solutions or lanolin preparations were used to treat one portion of a stem, 2,6-dichlorobenzoic acid traveled both upward and downward. The corresponding aldehyde derivative, however, traveled upward more readily than downward. The responses are indicated in Figure 1. When one branch of a Y-shaped plant was treated with the acid, the treated branch responded at the tip, but the material also moved down the stem, into the other branch, and upward causing swelling and modification of the leaves at the tip. In the case of the corresponding aldehyde, the treated branch responded but the other branch failed to respond within a 21-day period.

TABLE V

COMPARATIVE EFFECTS OF SUBSTITUTED BENZOIC ACIDS AND TWO CORRESPONDING ALDEHYDES FOR PHYSIOLOGICAL ACTIVITY WHEN APPLIED AT 0.1 PER CENT SPRAY SOLUTIONS

Substituents in nucleus of benzoic acid	Comparative effectiveness on tomato plants*		
	Cell elongation	Formative effects	Toxicity
None	o	o	
3-Amino	o	o	
4-Amino	o	o	
2-Chloro	o	o	
4-Chloro	o	o	
2-Iodo	o	++++	
3-Iodo	o	o	
4-Iodo	o	o	
2-Nitro	o	++++	
3-Nitro	o	o	+++
4-Nitro	o	o	++
2-Bromo-3-nitro	+++	+++	
2-Chloro-5-nitro	o	+++	
2-Chloro-6-nitro	o	o	
2,4-Dichloro	o	o	
2,5-Dichloro	++	++++	
2,6-Dichloro	++	+++	++++
(2,6-Dichlorobenzaldehyde)	o	+++	
3,5-Dinitro	o	o	++++
3-Amino-2,6-dichloro	o	o	
2-Amino-3,5-diiodo	o	+++	
4-Amino-3,5-diiodo	o	o	
2-Chloro-3,5-diiodo	+	+++	
2-Chloro-3,5-dibromo	+	+++	
2,6-Dichloro-3-nitro	+++	++++	
4,5-Dichloro-2-nitro	o	o	
2-Iodo-3,5-dibromo	o	++++	
2,3,6-Trichloro	++++	++++	
(2,3,6-Trichlorobenzaldehyde)	++++	++++	
2,3,5-Triiodo	+	++++	
2,3,4,5-Tetrachloro	o	o	

* o = Inactive; + = slight response; ++ = medium response; +++ = considerable response; ++++ = pronounced response.

There appeared, however, slight modification on the tip of the opposite branches six weeks after the treatment, indicating slight translocation in that direction. It was shown in an earlier publication (12) that 2,3,6-trichlorobenzoic acid and the corresponding aldehyde both moved freely upward and downward and were translocated readily to distant branches growing on the plant. Thus the similarity or difference in the translocation of an aldehyde and its corresponding acid depends upon the number, kind, and position of the substituents.

Stevia plants translocate some of the substituted benzoic acid and aldehyde derivatives more readily than the well-known root-inducing substances. The local effects of three of these are illustrated in Figure 2. It

FIGURE 1. Stevia plants used to illustrate translocation of material. Arrows indicate where preparations containing 25 mg. per g. of lanolin were applied. Left to right: Control; 2,6-dichlorobenzoic acid; 2,6-dichlorobenzaldehyde. Note that the acid traveled upward and downward moving into the opposite branch while the aldehyde appears to have moved only upward in the branch which was treated.

FIGURE 2. Stevia plants to show the local effect, indicated by arrows, of preparations containing 25 mg. of well-known root-inducing substances per g. of lanolin. Left to right: Control; indoleacetic acid; indolebutyric acid; naphthaleneacetic acid.

appears, therefore, that stevia varies considerably in its capacity to translocate various hormone-like substances.

DISCUSSION

By use of *Avena* coleoptile segments Muir and Hansch (7) found both 2-chlorobenzoic and 2,6-dichlorobenzoic acids active. The results obtained by the use of the tomato plant as the test object indicated that 2,6-dichlorobenzoic acid was active for both cell elongation and modification of leaves. The 2-chlorobenzoic acid, however, was inactive for both of these responses.

The activity of benzoic acid derivatives compared with the phenoxy derivatives shows some interesting differences. 2,6-Dichloro, 2,3,5-, 2,3,6-, 2,4,6-, and 3,4,5-trichlorophenoxyacetic acids are all inactive for cell elongation. However, of these, 2,6-dichloro, 2,3,4-, and 2,4,6-trichlorophenoxyacetic acids have formative effects, causing modification of the pattern of organs. After testing 15 compounds, Leaper and Bishop (4) showed that the highest physiological activity of chlorophenoxyacetic acids was associated with the presence of two unsubstituted positions in the benzene ring para to each other. Hansch and co-workers (2, 7) postulated an "ortho reaction hypothesis" to explain the activity of substituted ben-

zoic acids. They concluded that only those benzoic acids with an electronegative atom or group capable of displacement by an electron-rich substrate in one or both ortho positions can promote cell elongation of *Avena* coleoptile sections. They also describe a mechanism of reaction of the growth regulator with the plant substrate based upon electronic characteristics of active benzoic acids and phenoxyacetic acids.

In arriving at an explanation of how a plant growth regulant works, we are confronted with three or more well-known responses (cell elongation, cell division, induction of roots, and formative effects), all of which can be induced by a single substance, whereas some growth regulants cause only one or two of these responses. We are further faced with the problem that all species do not respond alike, as illustrated by the fact that *Avena* sections respond to treatment with 2-chlorobenzoic acid while tomato tissue does not. It would appear, therefore, that an explanation for one species may not serve for another.

SUMMARY

Evidence is presented to show that 2,6-dichlorobenzoic and 2,6-dichloro-3-nitrobenzoic acids induced cell elongation, cell division, adventitious roots, and formative effects on organs of plants. The corresponding aldehyde of 2,6-dichlorobenzoic acid caused formative effects but not the other responses listed for the acid. Stevia plants readily translocated the acid upward and downward while the corresponding aldehyde was translocated readily upward but either not at all or only slowly downward.

2,3,6-Trichlorobenzoic acid and the corresponding aldehyde caused similar responses when applied to tomato and stevia plants.

LITERATURE CITED

1. BENTLEY, JOYCE A. Growth-regulating effect of certain organic compounds. Nature [London] **165**: 449. 1950.
2. HANSCH, CORWIN, ROBERT M. MUIR, and ROBERT L. METZENBERG, JR. Further evidence for a chemical reaction between plant growth-regulators and a plant substrate. Plant Physiol. **26**: 812–821. 1951.
3. HUNTRESS, ERNEST HAMLIN. The preparation, properties, chemical behavior, and identification of organic chlorine compounds. 1443 pp. John Wiley & Sons, Inc., New York, N. Y. 1948.
4. LEAPER, J. M. F., and J. R. BISHOP. Relation of halogen position to physiological properties in the mono-, di-, and trichlorophenoxyacetic acids. Bot. Gaz. **112**: 250–258. 1951.
5. LEHMSTEDT, KURT, und KARL SCHRADER. Synthesen in der Acridonreihe (XVII Mitteil. über Acridin). Ber. Deutsch. Chem. Ges. **70B**: 1526–1538. 1937.
6. MEYER, VICTOR. Weiteres über die Esterbildung aromatischer Säuren. Ber. Deutsch. Chem. Ges. **28**: 182–189. 1895.
7. MUIR, ROBERT M., and CORWIN HANSCH. The relationship of structure and plant-growth activity of substituted benzoic and phenoxyacetic acids. Plant Physiol. **26**: 369–374. 1951.

8. NORDLANDER, B. W., and W. E. CASS. The esterification of tetrachlorophthalic anhydride. Jour. Amer. Chem. Soc. **69**: 2679–2682. 1947.
9. RUGGLI, PAUL, und HANS ZAESLIN. Über zwei neue Dichloro-o-nitro-benzoesäuren. Helvetica Chim. Acta. **19**: 434–439. 1936.
10. ZIMMERMAN, P. W. The formative influences and comparative effectiveness of various plant hormone-like compounds. Torreya **43**: 98–115. 1943.
11. ZIMMERMAN, P. W., and A. E. HITCHCOCK. Substituted phenoxy and benzoic acid growth substances and the relation of structure to physiological activity. Contrib. Boyce Thompson Inst. **12**: 321–343. 1942.
12. ———— Growth-regulating effects of chlorosubstituted derivatives of benzoic acid. Contrib. Boyce Thompson Inst. **16**: 209–213. 1951.

AN ESTIMATE OF THE QUANTITY OF ORAL SECRETION DEPOSITED BY LYGUS WHEN FEEDING ON BEAN TISSUE[1]

FLORENCE FLEMION, LAWRENCE P. MILLER, AND RICHARD M. WEED

Injury to plant tissue following feeding by *Lygus oblineatus* Say (tarnished plant bug) is so extensive that it is logical to presume that a toxic secretion may be involved. In recent experiments it was shown, with the use of P^{32}, that some radioactivity is left behind in bean tissue after the insects had fed (2). It has now been possible to estimate the quantity of secretion left in host tissue since saliva could be collected from the insects using a technique devised by Braun and Maramorosch (1)[2]. With the use of specially constructed micropipettes[3] volumes as low as 0.01 lambda could be measured and, with insects containing large amounts of P^{32}, the activity per unit volume could be determined. Thus by relating the amount of activity imparted to host tissue and the activity per unit volume of saliva of the insect concerned, a fairly good estimate of the quantity of secretion deposited could be made.

The insects were made highly radioactive by having them feed on sucrose solutions containing relatively large amounts of P^{32} using procedures previously described (2)[4]. It was necessary for the insects to take up amounts of P^{32} equivalent to about 500,000 counts per minute per insect, with measurement techniques detecting about 20 per cent of the disintegrations, in order to obtain saliva sufficiently active to be useful in these studies.

In using the technique of Braun and Maramorosch, only the upper part of the wings was attached to a glass slide with Duco cement and it was possible to free the insects again after salivation. Figure 1 A shows an insect attached to a slide and the tip of the micropipette near the proboscis. For the actual collection of saliva, the pipette was held at an angle other than that illustrated. When the insects salivated, a small droplet appeared at the end of the proboscis and was picked up by a micropipette which

[1] These results were presented before the New York Section of the American Chemical Society and an abstract has appeared in Amer. Chem. Soc., Absts. papers 4th Meeting-in-Miniature, New York, N. Y., Feb. 8, 1952, p. 4.

[2] The authors are indebted to Dr. Armin C. Braun for demonstrating this technique to them before the publication became available.

[3] The micropipettes used were made from precision bore capillary tubes by Karl Schumann, Cliffside, N. J.

[4] The P^{32} used in these experiments was obtained from the United States Atomic Energy Commission, Oak Ridge, Tenn.

Copyright, 1952, by Boyce Thompson Institute for Plant Research, Inc.

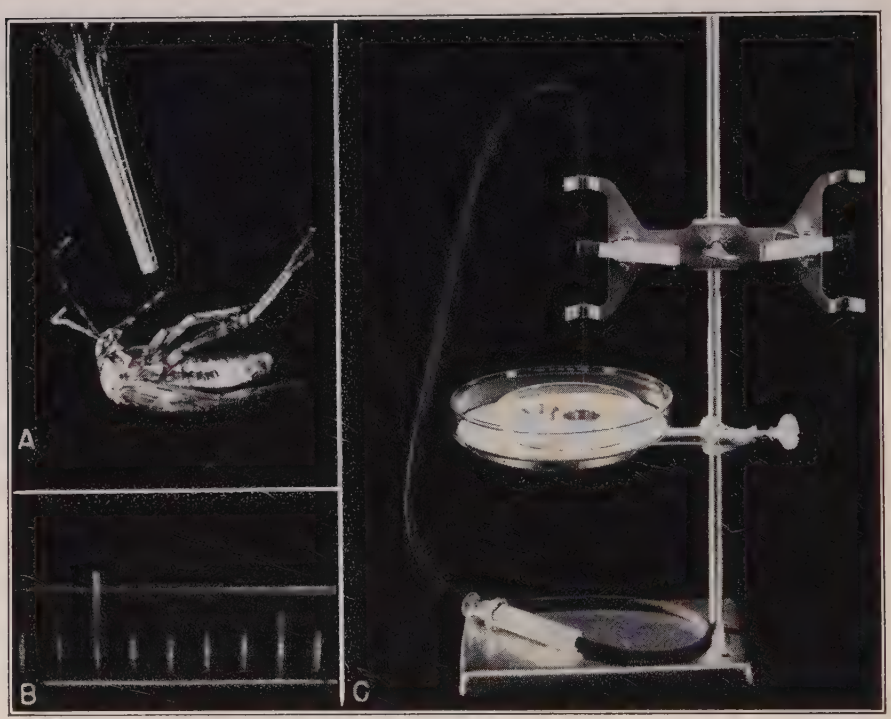


FIGURE 1. A. *Lygus* attached to a glass slide together with the tip of micropipette used for collecting saliva (magnification ×5). B. View of bore and calibration marks on pipette (magnification ×5). C. Illustration of method used in transferring material from pipette to stainless steel planchets prior to determination of radioactivity.

had previously been allowed to take up distilled water to fill the non-calibrated portion at the tip. These manipulations with the insects were carried out under the microscope at low magnification. The collected saliva was then transferred to a small stainless steel planchet with the aid of a short length of rubber tubing and a hypodermic syringe as shown in Figure 1 C. The pipette was repeatedly rinsed with distilled water to wash out the saliva as thoroughly as possible. In Figure 1 B is shown the pipette with the calibration marks at a magnification of ×5. The diameter of the bore of capillary as supplied by the manufacturer was stated to be 0.226 millimeter. When measured under the microscope a value of 0.23 millimeter was obtained. The volume of the pipette was also checked by using solutions containing radioactive phosphorus. Each scale division in the pipette corresponds to a volume of 0.04 lambda or microliter.

The insects, after feeding on the sucrose solution to which P^{32} had been added, were kept for a number of days on green bean (*Phaseolus vulgaris* L.) pod tissue before being used for the deposition experiments. This

procedure assured removal of any radioactivity that might have become associated mechanically with the mouth parts while ingesting the radioactive solution. When ready for the feeding experiments the insects were transferred to bean tissue prepared as described in a previous paper (2). The insects were kept under observation while feeding and transfer of radioactivity through excrement was thus avoided. After the insects had fed on the bean pods, the radioactivity of the tissue fed upon was determined. A sample of saliva was obtained from the insects as soon as possible after feeding and the radioactivity per unit volume determined.

The results obtained with collections of saliva from a number of insects which had taken in large amounts of radioactive P^{32} are shown in Table I. Values given in column two for the radioactivity of the insects are

TABLE I

RADIOACTIVITY PER UNIT VOLUME OF SALIVARY SECRETIONS OF LYGUS WHICH HAVE INGESTED P^{32}

Radioactivity of insects		Saliva collected		
Time after ingesting P^{32}, days	Counts per minute, $\times 10^{-3}$	Vol., lambda	Counts per minute and standard deviation	
			Collected sample	Per lambda
7	196	0.040	13 ± 3.9	325 ± 20
13	117	0.040	10 ± 2.4	250 ± 12
7	154	0.010	13 ± 2.8	1300 ± 28
10	117	0.012	12 ± 2.4	1000 ± 22
14	77	0.060	10 ± 2.9	167 ± 12
6	140	0.020	8 ± 3.7	400 ± 26
7	135	0.020	13 ± 2.0	650 ± 14
8	114	0.020	10 ± 1.6	500 ± 11
7	119	0.030	4 ± 2.0	133 ± 12
7	63	0.160	17 ± 3.6	105 ± 9
9	52	0.060	4 ± 2.1	66 ± 9

measurements made on the day the saliva was taken. The activity immediately after the end of the feeding period, from 6 to 14 days earlier was, of course, much higher. In the interim, losses as a result of decay and the voiding of radioactive excrement had taken place. Volumes of saliva collected at one time varied from 0.010 to 0.160 lamdba. Activities of saliva ranged from 66 to 1300 counts per minute per lambda. While the lower values were to some extent associated with insects of low total activity, there also is some indication that the amount of radioactivity per lambda was related to the total volume of saliva collected per insect. Thus the degree of hydration affected the amount of dilution involved and thereby influenced the radioactivity per unit volume.

To determine the volume of saliva deposited in host tissue, insects which had injested radioactive phosphorus were permitted after a waiting period to feed on green bean pod tissue. Times of feeding which were determined in each instance, varied from about 20 to approximately 108 minutes. The radioactivity imparted to tissue fed upon is shown in column three of Table II. Subsequent to feeding, saliva was collected directly from the insects.

TABLE II

DETERMINATION OF QUANTITY OF ORAL SECRETION DEPOSITED BY LYGUS WHEN FEEDING ON BEAN TISSUE

Radioactivity of *Lygus*		Radioactivity of bean pod tissue after feeding	Saliva collected after feeding		Calculated amount deposited in tissue, lambda
Time after ingesting P^{32}, days	Counts per minute, $\times 10^{-3}$	Counts per minute and standard deviation	Vol., lambda	Counts per minute and standard deviation	
7	162	60 ± 2.3	0.010	11 ± 2.4	0.055
9	147	62 ± 4.7	0.100	79 ± 4.8	0.078
7	144	38 ± 2.6	0.020	6 ± 2.8	0.063
6	140	15 ± 3.7	0.020	8 ± 3.6	0.038
9	135	46 ± 3.6	0.060	11 ± 4.0	0.251
13	77	13 ± 4.3	0.060	10 ± 2.9	0.078

The volumes of saliva and the radioactivity of the saliva are given in columns four and five of Table II. The calculated volume of the saliva deposited is given in column six of the table. Volumes ranged from 0.038 to 0.251 lambda. These volumes are higher than were obtained in the direct collections of saliva from the insects which is understandable in view of the fact that the collections involved saliva appearing at the tip of proboscis within relatively short periods while feeding times on host tissue were as long as 108 minutes.

In these tests insects having an activity of from 77,000 to 162,000 counts per minute imparted activities of 13 to 62 counts per minute to bean tissue. These values represent dilution factors of about 3,000 to 9,000, values which are close to those previously obtained (2). Times of feeding were observed with all the tests reported in Table II but the amount of activity imparted was not directly associated with the length of feeding time. Apparently the insects are not necessarily actually feeding during the whole time that the proboscides are inserted into the host tissue so that the observed times are not really true measurements of total feeding time.

Data reported in Tables I and II represent only those instances in which significant quantities of saliva were obtained. For reasons not clear at this time salivation took place in only about 50 per cent of the insects subjected to this procedure.

SUMMARY

An estimate of the quantity of oral secretion deposited in green bean pod tissue by *Lygus oblineatus* on feeding was obtained through the aid of radioactive phosphorus. With the use of a technique developed by Braun and Maramorosch saliva was collected from *Lygus* which had fed on P^{32} and the activity per unit volume was determined. On the basis of the radioactivity imparted to bean tissue by insects on feeding, it was estimated that from 0.05 to 0.25 lambda was deposited in the tissue as a result of feeding periods varying from about 20 to 108 minutes.

LITERATURE CITED

1. BRAUN, ARMIN C., and KARL MARAMOROSCH. A method for obtaining saliva from certain leafhoppers. Phytopath. **41**: 1126–1128. 1951.
2. FLEMION, FLORENCE, RICHARD M. WEED, and LAWRENCE P. MILLER. Deposition of P^{32} into host tissue through the oral secretions of *Lygus oblineatus*. Contrib. Boyce Thompson Inst. **16**: 285–294. 1951.

BIOLOGICAL ACTIVATION OF SODIUM 2-(2,4-DICHLOROPHENOXY)ETHYL SULFATE[1] BY BACILLUS CEREUS VAR. MYCOIDES

A. J. Vlitos[2]

Sodium 2-(2,4-dichlorophenoxy)ethyl sulfate has been shown to require contact with soil before its hormonal action on plants can be manifested (3). A microbial factor was considered necessary for the chemical conversion by King, Lambrech, and Finn (3), and this has since been confirmed (1, 4). Carroll (1) reported that acid hydrolysis of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate in solutions of low pH resulted in the formation of 2,4-dichlorophenoxyethanol and sodium acid sulfate. The compound was also readily converted to its active form in nonsterile or sterile soils of low pH values (4). Activation did not occur in sterile soil if the hydrogen ion concentration was above pH 5.5, whereas in nonsterile soil the conversion to an active compound occurred in a pH range of 4.0 to 7.0. Activation did not occur at a pH of 8.0.

The present paper is concerned chiefly with the isolation from soil of organisms effective in converting sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to an active plant growth regulator. Preliminary exeriments concerning the pathway of the chemical steps involved in the activation are also discussed.

MATERIALS AND METHODS

Sandy loam soil (pH 5.5 to 7.0) was treated with 100 p.p.m. of the herbicide in Petri dishes and allowed to stand at 21° C. for seven days. At the end of this incubation period soil dilution plates were prepared as follows: ten gram samples of the treated soils were added to 200 ml. of sterile water in 500 ml. Erlenmeyer flasks, which were then shaken for thirty minutes on an automatic "wrist-action" shaker. After removal from the shaker, the soil-water mixture in each flask was allowed to stand for ten minutes until heavier soil particles settled to the bottom.

From the original 1:20 dilutions, serial dilutions were prepared. Differential media were employed, consisting of soil agar (2) for actinomycetes, nutrient agar for bacteria, and potato dextrose agar for fungi. One ml. of a 1:100,000 dilution was pipetted to each of three sterile Petri plates, and cool agar poured over the dilution. All cultures were incubated at room temperature (20° C. to 25° C.). After 24 hours, transfers were made of the

[1] Sold under the trade name CRAG Herbicide 1.

[2] Senior Fellow in Plant Physiology, Boyce Thompson Institute for Plant Research, Inc., Yonkers 3, N. Y., and the Carbide and Carbon Chemicals Company, New York 17, N. Y.

bacterial colonies appearing on the Petri plate cultures to test tube slants containing nutrient agar. Similar transfers were made after four or five days of fungus and actinomycete colonies to test tube slants of potato dextrose and soil agar. When the soil organisms were well established in test tube culture, each of the isolates was tested for its ability to convert sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to its active form in sterile soil.

A modified cucumber root suppression test (3) was employed to measure activation of the compound. Five grams of soil, similar to the original soil from which the isolations were made, were treated with 5 p.p.m. of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate in Petri plates. Sterilization was achieved by autoclaving the soil-herbicide mixture at 15 lb. pressure for 30 minutes. Previous experiments had shown that the chemical was not altered by this treatment. Each of the sterile soil-herbicide mixtures was then inoculated with 1 ml. of a water suspension of each isolate. Twenty-five cucumber (*Cucumis sativus* L. var. Davis' Perfect) seed were placed in contact with the soil-herbicide-microbial mixture and incubated for five days at 21° C. Controls consisting of sterile soil, herbicide, and soil isolate were included. Measurements of the primary roots were made after five days.

DISCUSSION AND RESULTS

Of the isolations made, bacteria were found to be most active in converting sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to its active form. In the initial experiments no effort was made to identify each isolate. However, if the isolate exhibited activity it was then taxonomically determined. Representative data from a portion of soil isolate-herbicide activation tests appear in Table I.

TABLE I

EFFECT OF SOME BACTERIAL ISOLATES IN ACTIVATING SODIUM 2-(2,4-DICHLOROPHENOXY)ETHYL SULFATE IN STERILE SOIL

Na 2-(2,4-dichlorophenoxy)-ethyl sulfate, 5 p.p.m.	Bacterial isolate	Cucumber root length,* cm.
Present	None	2.1
Absent	None	2.2
Present	B-1	0.5
Absent	B-1	2.1
Present	B-11	0.7
Absent	B-11	2.5
Present	B-10	2.1
Absent	B-10	2.5

* Average primary cucumber root length for 50 seed.

The responses of cucumber primary roots to the activated form of the herbicide induced by pure cultures of B-1 and B-11 were the typical swelling and marked stunting of growth. These qualitative manifestations con-

firmed the quantitative root length measurements. Culture B-1 was identified as *Bacillus cereus* var. *mycoides* (Flügge) Smith, Gordon, and Clark. Standard bacteriological procedures were followed for identification of the organism. Results of these may be summarized as follows: gram-positive rods 1.0 micron to 1.2 microns by 3.0 microns to 5.0 microns; rhizoidal growth on nutrient agar slants; loss of rhizoidal characteristic in nutrient broth; gelatin liquefaction rapid; negative fermentation tests on xylose and arabinose; positive on sucrose and glucose; positive Voges-Proskauer reaction; positive starch hydrolysis; nitrites produced from nitrates.

The nature of the bacterial action on sodium 2-(2,4-dichlorophenoxy)-ethyl sulfate and the steps involved in its transformation to an active plant growth regulator were also investigated. One of the first questions that arises in such a study is whether the transformation is accomplished within, or external to, the bacterial cell. This was studied by obtaining cell-free filtrates of nutrient broth in which *Bacillus cereus* var. *mycoides* had been grown for 24 hours. Broth cultures of the organism were passed through a Morton bacteriological filter. The resulting filtrate was tested for sterility by transfer of a drop to a nutrient agar slant. These cell-free preparations were then tested for their action on sodium 2-(2,4-dichlorophenoxy)ethyl sulfate. Results obtained in such an experiment are listed in Table II.

TABLE II

EFFECT OF CELL-FREE FILTRATES OF BACILLUS CEREUS VAR. MYCOIDES IN ACTIVATING SODIUM 2-(2,4-DICHLOROPHENOXY)ETHYL SULFATE

Treatment	Concentration of chemical, p.p.m.	Cucumber root length,* cm.
Filtrate+chemical	5	0.6
	0.5	1.0
	0.05	1.2
Chemical alone	5	2.3
	0.5	2.2
	0.05	2.3
Autoclaved filtrate+chemical	5	2.5
	0.5	2.3
	0.05	2.5
Filtrate alone	—	2.0
Water control	—	2.2

* Average primary cucumber root length for 50 seed.

Since the pH of the filtrates used above was established to be 8.3 to 8.5 acid hydrolysis obviously cannot be considered the significant reaction in this case. The chemical was converted to its active form when it came into contact with filtrates of nutrient broth cultures of *Bacillus cereus* var.

mycoides, but conversion did not occur if these filtrates were previously autoclaved for 15 minutes at 15 lb. pressure. Since neither the filtrate alone nor the chemical alone suppressed cucumber root elongation, it is concluded that a heat labile substance, secreted by *Bacillus cereus* var. *mycoides* into the nutrient broth medium, is capable of converting sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to an active herbicide. It is possible that an enzymatic hydrolysis accounts for this conversion. This possibility is presently being investigated and should yield considerable information concerning the action of soil microbes on sodium 2-(2,4-dichlorophenoxy)-ethyl sulfate.

SUMMARY

Bacillus cereus var. *mycoides* (Flügge) Smith, Gordon, and Clark is effective in converting sodium 2-(2,4-dichlorophenoxy)ethyl sulfate to an active plant growth regulator.

Cell-free filtrates of nutrient broth cultures of the organism were capable of causing the conversion. Autoclaved cell-free filtrates were incapable of activating the compound. Apparently a heat labile substance, secreted by *B. cereus* var. *mycoides* into the nutrient broth medium, can cause biological activation of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate. The enzymatic nature of this activation is suggested.

LITERATURE CITED

1. CARROLL, R. B. Factors influencing the activation of 2,4-dichlorophenoxyethyl sulfate. Proc. Southern Weed Conf. **4**: 13. 1951.
2. HOOKER, W. J. Parasitic action of *Streptomyces scabies* on roots of seedlings. Phytopath. **39**: 442–462. 1949.
3. KING, LAWRENCE J., J. A. LAMBRECH, and THOMAS P. FINN. Herbicidal properties of sodium 2,4-dichlorophenoxyethyl sulfate. Contrib. Boyce Thompson Inst. **16**: 191–208. 1950.
4. VLITOS, A. J. The influence of environmental factors on the activity of CRAG Herbicide 1 (sodium 2,4-dichlorophenoxyethyl sulfate). Proc. Northeastern Weed Control Conf. New York City, Jan. 1952. pp. 57–62.

DETECTION AND DETERMINATION OF 2,4-D AS A CONTAMINANT BY BIOLOGICAL METHODS

P. W. Zimmerman, A. E. Hitchcock, and Henry Kirkpatrick, Jr.

2,4-Dichlorophenoxyacetic acid (2,4-D) is one of the most active plant growth regulants in use today. Because of its high degree of toxicity 2,4-D is commonly used as a herbicide. When being applied to weeds, accidental injury to neighboring crops frequently results from vapor or spray drift. Damage has also occurred where insecticides and fungicides were accidentally contaminated with 2,4-D. Contamination can result from the use of 2,4-D spraying equipment which was not thoroughly cleaned before being used for insecticide or fungicide applications. It can also result from the use of reclaimed 2,4-D drums, not completely cleaned of 2,4-D, for packaging or mixing insecticide or fungicide formulations. Contamination is known to have resulted from storing volatile esters of 2,4-D together with agricultural formulations of insecticides, fungicides, and fertilizers. The cause of damage to crops is frequently difficult to diagnose and may call for special techniques.

The purpose of this report is to describe the methods and results of biological tests involving the detection of minute amounts of 2,4-D alone and in known and unknown mixtures with other substances.

MATERIALS AND METHODS

Chemicals. The following compounds and formulations were used: the triethanolamine salt, and the isopropyl, butyl, and polyethylene glycol esters of 2,4-D; the triethanolamine salt of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T); 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT); *O,O*-diethyl *O*-*p*-nitrophenyl thiophosphate (parathion); and chlorinated camphene (toxaphene). These materials were used alone and in mixtures with 2,4-D. Concentrations of 2,4-D and 2,4,5-T are expressed as percentages of the free acid equivalent on a volume basis. Concentrations of the other materials are expressed as percentages of the materials on a volume basis.

Test plants. Greenhouse-grown Bonny Best tomato plants (*Lycopersicon esculentum* Mill.) four to five inches in height with four well-developed leaves were used as the standard test plants. Greenhouse-grown Delta Pine cotton (*Gossypium hirsutum* L.) plants at various stages of growth were included in some of the tests as representative of a crop plant especially sensitive to 2,4-D. Numerous other plant species, including weed, woody, and crop plants which received doses of 2,4-D, are referred to in this report.

Application techniques. Materials were applied to the test plants as

Copyright, 1952, by Boyce Thompson Institute for Plant Research, Inc.

aqueous solutions, as vapors, or in lanolin. The aqueous solutions were applied as sprays or as small measured drops according to the technique of Hitchcock and Zimmerman (3). The sprays were applied to the test plants with a deVilbiss hand atomizer at concentrations ranging from 0.0001 per cent to 10 per cent. In the case of the tomato test plant approximately 3 ml. of solution were applied per plant. Each preparation was applied to two or more plants. Measured volumes (0.01 ml.) of a test solution were applied to one or more leaflets or leaves of the test plants by means of a graduated 0.1 ml. pipette. The concentrations of the test solutions ranged from 0.001 per cent to 10 per cent, and the amount applied per plant ranged from 0.1γ to 1000γ. Applications of aqueous solutions of the materials to the root systems of the test plants were made by adding 20 ml. of solution per four-inch pot. Concentrations of the solutions used for soil applications ranged from 0.00001 per cent to 1 per cent.

Measured amounts of the materials were mixed with measured amounts of lanolin and then applied by means of a glass rod to the stem or petiole, or to both, of the test plant. In the case of the tomato plant the lanolin mixture was applied to the upper surface of the petiole of the third or fourth leaf above the base, and to the adjoining stem area. Concentrations of the materials in the lanolin ranged from 0.01 mg. per gram (0.001 per cent) to 10 mg. per gram (1 per cent).

Vapors were applied to the test plants by placing 1 or 2 ml. of the test material on a watch glass under a bell jar with the plant. Exposures were for 2 hours at temperatures ranging from 110° to 120° F. and for 16 hours at temperatures ranging from 70° to 80° F. Considerable difficulty was encountered in cleaning the bell jar after exposure to high volatile esters. Therefore, a bag technique (1) was used wherein five 0.01 ml. drops of the test material were added to filter paper, and this, together with the test plant, was enclosed in a paper bag for 16-hour exposures at 70° to 80° F. and for 2-hour exposures at 110° to 120° F. Control plants were enclosed in paper bags in a similar manner except that 0.05 ml. of distilled water was added to the filter paper. In addition, uncovered control plants were placed next to the sealed bags. After treatment each bag was carried to the laboratory hood where the plant was removed and the bag was destroyed. Treated plants not showing curvature responses within 48 hours were transferred to the greenhouse for further observation, particularly with reference to the development of modified leaves. This method reduces the possibility of contamination which frequently occurs with bell jars.

RESPONSE OF TEST PLANTS

The various formative effects induced by 2,4-D on tomato plants depended upon the concentration, age of the plant, and the method of application (Table I). Many other chemical compounds induce formative re-

TABLE I

THRESHOLD CONCENTRATIONS (PER CENT) OF 2,4-D FOR INDUCING DIFFERENT RESPONSES ON TOMATO PLANTS BY VARIOUS METHODS OF TREATMENT

Responses	Methods of application			
	Foliage spray	0.01 Ml. drop	Lanolin	20 Ml. soil
Modification	0.0001	0.001	0.001	0.0001
Leaf bending	0.0005	0.005	0.01	0.0001
Stem bending	0.001	0.01	0.01	0.001
Proliferation	0.005	0.025	0.1	0.01
Stunting	0.01	0.1	0.1	0.01
Killing	0.1	10.0	1.0	0.1

sponses on tomato plants which might be confused with those induced by 2,4-D. Some of these are listed in Table II. Substituted phenoxy and benzoic acids are particularly effective for inducing formative effects involving modification of the pattern of leaves, stems, and fruit (5, 6). The two growth-regulating compounds most widely used today as herbicides are 2,4-D and 2,4,5-T. 2,4-D is the most active when considered from a concen-

TABLE II

EXAMPLES OF ACTIVE AND INACTIVE SUBSTANCES FOR MODIFICATION OF TOMATO LEAVES

Active acids	Inactive acids and insecticides
α-Naphthoxyacetic	Indoleacetic
β-Naphthoxyacetic	Indolebutyric
o-Chlorophenoxyacetic	α-Naphthaleneacetic
p-Chlorophenoxyacetic	β-Naphthaleneacetic
2,4-Dichlorophenoxyacetic	Monochloroacetic
2,4,6-Trichlorophenoxyacetic	*o*-Chlorophenoxypropionic
2,5-Dichlorobenzoic	2,4-Dichlorophenoxypropionic
2,6-Dichlorobenzoic	2,4,5-Trichlorophenoxyacetic
2,3,5-Triiodobenzoic	2,4-Dichlorophenoxyethyl sulfate**
2,3,6-Trichlorobenzoic	DDT
Maleic hydrazide*	Parathion
	Toxaphene

* Diethanolamine salt.
** Active for modification only when applied in the presence of soil.

tration standpoint. 2,4,5-T differs from 2,4-D in that it does not cause modification of leaves of tomato and cotton. Both substances cause cell elongation resulting in stem and leaf curvatures, proliferation of the stem, growth inhibition, and killing if the dose is great enough (Fig. 1). The modification induced on cotton plants (Fig. 2) by 2,4-D resembled that induced on tomato (Fig. 3 A) in that the leaflets or leaves were dwarfed, the venation became pronounced due to clearing of the veins, and the marginal leaf pattern varied from the normal. Parathion, DDT, and toxa-

FIGURE 1. Response of Bonny Best tomato plants to known concentrations of 2,4-D and to insecticides contaminated with 2,4-D. Appearance of plants two days after spraying with (left to right) (A) 0, 0.0001, 0.001, 0.01, and 0.1 per cent 2,4-D; and (B) 0, 0.001 per cent 2,4-D, toxaphene-DDT formulation diluted to 1 per cent, toxaphene-DDT formulation diluted to 5 per cent, and 0.01 per cent 2,4-D.

phene alone induced no growth regulatory or formative effects on either tomato or cotton plants. Undiluted preparations of all three substances, however, were toxic when applied to leaves. The responses induced by mixtures containing 2,4-D and either DDT, parathion, or toxaphene were the same as those induced by 2,4-D alone, showing that the insecticides did not affect the capacity of 2,4-D to induce formative effects.

Vapors from the isopropyl or the butyl esters of 2,4-D induced the same type of formative effects on the test plants as those induced when 2,4-D acid or salt were applied by other methods. The volatility varied with the form of 2,4-D used and the temperature at the time of treatment. Exposure of the plants for 24 hours at 70° to 80° F. to vapors from the isopropyl or the butyl ester induced pronounced formative effects as compared to none induced by vapors from the acid, triethanolamine salt, or polyethylene glycol ester for the same length of exposure. Exposure for 2 hours at 110° to 120° F. to vapors from the acid or the triethanolamine salt of 2,4-D did not induce any formative effects. Slight formative effects were induced by the polyethylene glycol ester at the higher temperatures. However, the degree of response was considerably less than that induced by the isopropyl or butyl ester at the lower temperatures. Vapors from DDT, parathion, or toxaphene induced no formative effects on the test plants.

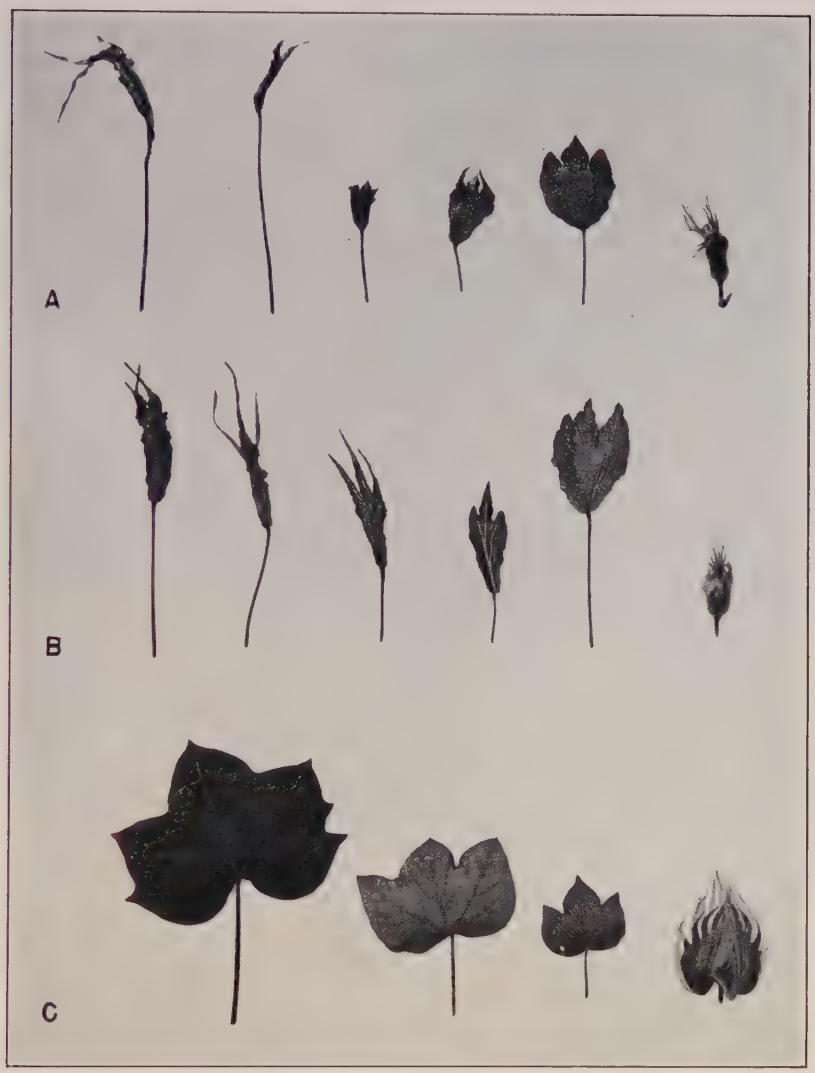


FIGURE 2. Leaves and flower bud of Delta Pine cotton plants three weeks after spraying with (A) toxaphene-DDT formulation (contaminated with 2,4-D) diluted to 5 per cent; (B) 0.001 per cent 2,4-D; and (C) non-treated controls.

FIGURE 3. Effect of 2,4-D on tomato and cotton. A. Normal tomato leaf at left with increasing degrees of modification up to a pronounced type at right. B. Four-week responses induced by 2,4-D sprays on two-month-old Delta Pine cotton plants. Left to right: control, 0.001, 0.01, and 0.1 per cent.

FIGURE 4. Modified leaves induced by 2,4-D spray drift. A. Normal leaves above and modified leaves below. Left to right: A. *Rubus* sp., *Fraxinus americana* L., *Eupatorium purpureum* L., and *Vitis* sp. B. Normal leaves on left, modified leaves on right. *Cornus florida* above, and *Ailanthus glandulosa* below.

TABLE III

SPECIES OF PLANTS SHOWING LEAF MODIFICATION AFTER EXPOSURE TO SUB-LETHAL DOSES OF 2,4-D

Herbaceous weeds	Cultivated annuals	Woody plants
Bindweed (*Convolvulus* sp.)	Bean (*Phaseolus vulgaris* L.)	Apple (*Pyrus malus* L.)
Cocklebur (*Xanthium spinosum* L.)	Cosmos (*Cosmos bipinnatus* Cav.)	Ash (*Fraxinus americana* L.)
Dandelion (*Taraxacum officinale* Weber)	Cotton (*Gossypium hirsutum* L.)	Birch (*Betula* sp.)
Dock (*Rumex* sp.)	Cucumber (*Cucumis sativus* L.)	Blackberry (*Rubus* sp.)
Hawkweed (*Hieracium* sp.)	Eggplant (*Solanum melongena* L.)	Dogwood (*Cornus florida* L.)
Joe-Pye-weed (*Eupatorium purpureum* L.)	Marigold (*Tagetes erecta* L.)	Elderberry (*Sambucus canadensis* L.)
Mallow (*Malva* sp.)	Okra (*Hibiscus esculentus* L.)	Elm (*Ulmus* sp.)
Nightshade (*Solanum* sp.)	Petunia (*Petunia axillaris* Lam.)	Grape (*Vitis* sp.)
Plantain (*Plantago* sp.)	Phlox (*Phlox paniculata* L.)	Honeysuckle (*Lonicera japonica* Thunb.)
Pokeweed (*Phytolacca decandra* L.)	Potato (*Solanum tuberosum* L.)	Maple (*Acer* sp.)
Ragweed (*Ambrosia* sp.)	Sunflower (*Helianthus annuus* L.)	Mulberry (*Morus* sp.)
Self-heal (*Prunella vulgaris* L.)	Tomato (*Lycopersicon esculentum* Mill.)	Poison ivy (*Rhus toxicodendron* L.)
	Zinnia (*Zinnia* sp.)	Rose (*Rosa* sp.)
		Sumac (*Rhus* sp.)
		Tree of Heaven (*Ailanthus glandulosa* Desf.)
		Trumpet creeper (*Campsis radicans* Seem.)
		Willow (*Salix* sp.)
		Wisteria (*Wisteria* sp.)

Many other species of plants showed formative effects when treated with 2,4-D (Fig. 4). Some of the more common crop, weed, and woody plants that are sensitive to 2,4-D are listed in Table III.

The decreasing order of sensitivity for the different responses was: leaf modification, leaf bending, stem bending, stem proliferation, and killing. The degree of response was dependent upon the dose of 2,4-D applied, and this dose was determined by the concentration and the method of application (Table IV). For example, it required a minimum dose of approximately 3 ml. of a 0.0001 per cent 2,4-D solution which is equivalent to 3γ

TABLE IV

DEGREE OF RESPONSE INDUCED BY DIFFERENT AMOUNTS OF 2,4-D APPLIED TO ONE LEAFLET OF A TOMATO PLANT

γ2,4-D per plant	Relative degree of response					
	Modification	Leaf bending	Stem bending	Proliferation	Stunting	Killing
1000	o	+++++	+++++	+++++	++++	9–10 days
100	o	++++	++++	+++++	+	o
10	++	+++	+++	++++	o	o
1	++++	+	+	o	o	o
0.1	+	o	o	o	o	o

+ = Trace, ++ = slight, +++ = moderate, ++++ = pronounced, +++++ = very pronounced.

of 2,4-D to induce modification on a tomato plant when the material was applied as a foliage spray. In contrast, it required only 0.1γ of 2,4-D to induce similar modification when a 0.01 ml. drop of a 0.001 per cent 2,4-D solution was used. With the soil method of application it required 20 ml. of a 0.0001 per cent 2,4-D solution or 20γ of 2,4-D to induce modification.

Cotton plants showed practically the same degree of sensitivity to 2,4-D as tomato plants. Effects of 2,4-D sprays applied at concentrations ranging from 0.0001 per cent to 0.1 per cent to three-month-old Delta Pine cotton plants varied from leaf modification induced by the lowest concentration to severe injury induced by the highest concentration. 2,4-D induced modification of organs, stem proliferations, stunting, leaf and bud injury, and killing on both cotton (Fig. 3 B) and tomato plants. Growth of cotton plants was inhibited within a few days after treatment by a 0.001 per cent 2,4-D spray, and flower buds were killed within 10 days if sprayed with a 0.01 per cent 2,4-D spray. A 0.001 per cent 2,4-D spray on immature squares of cotton resulted in malformed flowers and bolls. Cotton seedlings treated by the drop method with 1γ of 2,4-D developed very pronounced leaf modification within one month. All growth responses except modification of organs were induced on cotton plants by various doses of 2,4,5-T.

Surveys of vegetation were made in various sections of the country when it was suspected that plants had been accidentally exposed to 2,4-D. Responses similar to those induced by known 2,4-D applications were found on plants not intentionally treated with 2,4-D growing in agricultural areas (cotton fields, sugar plantations, and tomato fields), around factories formulating 2,4-D herbicides, and in cities and towns where 2,4-D herbicides were used in weed control programs. The growth responses resulted from 2,4-D contamination of insecticide formulations during manufacture, formulation, storage, or application (including spraying equipment), from 2,4-D spray drift during spraying operations in nearby areas, or from air pollution caused by 2,4-D vapor.

DISCUSSION

It has been reported that a number of chemical compounds, when applied in mixtures with 2,4-D, reduced or increased the effectiveness of 2,4-D (3). Results in this report are concerned with equivalencies based on the degree of response induced by known amounts of 2,4-D alone as compared to the degree of response induced by 2,4-D in a mixture. A comparison of the responses induced by the 2,4-D on the test plants with those induced by the mixtures indicated that the responses of 2,4-D alone were similar to those of the 2,4-D induced by the mixtures (Fig. 1). It is the growth responses induced on the test plants with a mixture that are of practical importance. Amounts of 2,4-D as low as 0.0001 per cent in agricultural formulations were determined by the growth responses induced on

tomato and cotton plants. Modification of the pattern of leaves appears to be the most sensitive detectable response of plants to 2,4-D that can be measured quantitatively (3).

Damage resulting from spray drift can be minimized or prevented by the use of especially designed spray equipment and caution on the part of those applying 2,4-D sprays. For example, both the airplane and the helicopter are used in applying 2,4-D sprays. Results of applying 2,4-D sprays to water hyacinth (*Eichhornia crassipes* Solms.) by means of a helicopter were reported in 1950 (4, 7). Spray drift was limited to 25 feet or less due to the maneuverability of the helicopter and the downdraft (20 to 25 m.p.h.) created by the overhead rotor blades which forces the spray droplets downward. In areas where precise control of the spray pattern is demanded the helicopter would be preferable to the airplane. Regardless of the equipment used, wind conditions at the time of spraying, spraying pressure, and the rate of application (gallons per acre) used are important factors relating to the control of 2,4-D spray drift.

Damage resulting from vapor drift can be minimized or prevented by the use of low volatile forms of 2,4-D. For example, the acid, a salt, or a low volatile ester formulation should be used in preference to the highly volatile ester formulations in areas where other plants sensitive to 2,4-D are growing.

Damage resulting from 2,4-D contamination of insecticide or fungicide formulations can be minimized or prevented by avoiding the use of 2,4-D spraying equipment to apply an insecticide or a fungicide and empty 2,4-D drums or containers for packaging or mixing insecticide or fungicide sprays. It is difficult to wash all of the 2,4-D out of spraying equipment or metal containers.

When this manuscript was ready for the press, it came to our attention that Hall and Johnson (2) had reported on a bio-assay method to detect contamination of insecticides involving the cotton plant as a test object. Their findings and recommendations are similar to that portion of our paper involving the cotton plant.

SUMMARY

Methods involving the use of tomato and cotton plants are described for the detection and determination of 2,4-D in unknown solutions, mixtures of 2,4-D and other growth regulants, and commercial formulations. Materials were applied to test plants as aqueous solutions, as vapors, and in lanolin. The principal responses induced on test plants were modification of leaves, curvature of leaves and stems, proliferation and killing of tissue. The modification of leaves was the most sensitive response. Amounts of 2,4-D as low as 1 p.p.m. were detected in mixtures with 2,4,5-T, DDT, parathion, and toxaphene. Formulations of insecticides contaminated with

2,4-D induced the same responses on tomato and cotton plants as were induced by 2,4-D.

In practice the typical modification of leaves and other growth responses of many wild species and horticultural types were found useful in detecting the presence of 2,4-D. The source of contamination was traced to insecticides, spray equipment, polluted air around factories, warehouses, and where herbicides had been used.

LITERATURE CITED

1. DAINES, ROBERT H. 2,4-D as an air pollutant and its effect on various species of plants. *In* Air pollution. p. 140–143. McGraw-Hill Book Co., New York. 1952.
2. HALL, WAYNE C., and S. P. JOHNSON. A bio-assay for hormone contamination of insecticides. Texas Agric. Exp. Sta. Prog. Rept. 1421. 5 pp. Dec. 20, 1951.
3. HITCHCOCK, A. E., and P. W. ZIMMERMAN. A quantitative method of measuring response of plants to growth regulators. Contrib. Boyce Thompson Inst. **16**: 225–248. 1951.
4. HITCHCOCK, A. E., P. W. ZIMMERMAN, HENRY KIRKPATRICK, JR., and T. T. EARLE. Growth and reproduction of water hyacinth and alligator weed and their control by means of 2,4-D. Contrib. Boyce Thompson Inst. **16**: 91–130. 1950.
5. ZIMMERMAN, P. W. Formative effects of some hormone-like growth regulators. *In* Skoog, Folke, editor. Plant growth substances. p. 175–183. Univ. Wisconsin Press, Madison. 1951.
6. ZIMMERMAN, P. W., and A. E. HITCHCOCK. Substituted phenoxy and benzoic acid growth substances and the relation of structure to physiological activity. Contrib. Boyce Thompson Inst. **12**: 321–343. 1942.
7. ZIMMERMAN, P. W., A. E. HITCHCOCK, HENRY KIRKPATRICK, JR., and T. T. EARLE. Practical control of water hyacinth with 2,4-D. Agric. Chem. **5**(2): 45–47, 49, 81, 83–85. Feb., 1950. (*Also in* Boyce Thompson Inst. Prof. Pap. **2**: 75–86. 1950).

THE INSECTICIDAL ACTIVITY OF THE ETA ISOMER OF 1,2,3,4,5,6-HEXACHLOROCYCLOHEXANE

R. C. Back[1]

The announcement of the insecticidal properties of 1,2,3,4,5,6-hexachlorocyclohexane, hereafter referred to as benzene hexachloride, in 1945 (7) was accompanied by data on the relative activity of the four known stereoisomers. The fact that the gamma isomer was much more highly insecticidal than the alpha, beta, or delta isomers has been substantiated by numerous workers (1, 4, 5, 6). The fifth isomer, epsilon, was isolated in 1947 (2) and has since been found to be insecticidally inactive (5, 6). The recent discovery of the eta isomer by Kolka, Orloff, and Griffing (3) aroused interest in its insecticidal potentialities, and a few tests were made on the house fly, *Musca domestica*, L., the yellow-fever mosquito, *Aedes aegypti* (L.), the German cockroach, *Blatella germanica* (L.), and the confused flour beetle, *Tribolium confusum* Duv. The results of tests against these species with the alpha, gamma, delta, and eta isomers of benzene hexachloride, and with different insecticides, are presented in this report.

MATERIALS AND METHODS

The alpha, delta, and eta isomers of benzene hexachloride were supplied in pure form by the Ethyl Corporation. Lindane (gamma 1,2,3,4,5,6,-hexachlorocyclohexane), DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane], TEPP (tetraethyl pyrophosphate), and chlordane (1,2,4,5,6,7,8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene) were obtained from the Wisconsin Alumni Research Foundation as Standard Reference Insecticides of the American Association of Economic Entomologists. Reported assays on these materials were: Lindane, 100 per cent; DDT, 77.2 per cent (technical); TEPP, 40 per cent; and chlordane, 60 to 75 per cent (technical). Pyrethrum was obtained as a 20 per cent extract of pyrethrins in petroleum oil, while the technical grade benzene hexachloride was a laboratory sample of unknown origin.

Although only 300 mg. of eta isomer were available, it was desirable to evaluate this material against a representative group of insect species. For this reason small insect populations were used and few replications were made. Three tests were run on each species; tests included two to five concentrations of each test chemical.

Mosquito larva test. Fourth instar larvae were tested in distilled water

[1] Entomologist, Ethyl Corporation Project, Boyce Thompson Institute for Plant Research, Inc.

Copyright, 1952, by Boyce Thompson Institute for Plant Research, Inc.

to which the toxicants had been added as acetone solutions. The final test preparations contained not more than 1 per cent acetone, 25 larvae per replicate, two replicates per test, and were held at room temperature for 48 hours. Only those larvae unable to display any movement whatsoever were considered dead at the end of the exposure period. The check groups, which were treated with solutions of acetone in water equivalent to those used in the toxicant preparations, exhibited no mortality.

House fly test. Adult flies, five to seven days old, were exposed to residues obtained by evaporating acetone solutions of the toxicants on the inside of large test tubes (3×20 cm.). The flies were inactivated by cold during transfer to the tubes. Food was supplied as sugar solution in cotton-stoppered vials (1×8 cm.) which were wired in place inside the larger test tubes; cheesecloth tops completed the caging. The tubes were held in a ventilated laboratory hood for 24 hours. Flies unable to walk after this exposure were considered dead. Each test consisted of one replicate of each treatment using a sample size of 25 flies. There was no mortality in the treated controls.

Cockroach test. Adult males of the German cockroach, eight weeks old from hatching, were anaesthetized with carbon dioxide and dipped in the formulated toxicants. Formulations were prepared by adding an emulsifier[2] to acetone solutions of the test materials, which were then diluted with water so that the final acetone concentration never exceeded 5 per cent. In subsequent dilutions the emulsifier concentration was maintained at its original strength of 0.02 per cent. The dipping time was 10 to 15 seconds at 27° C. The roaches were maintained at 27° C. in open containers without food or water for 48 hours. Insects which were unable to demonstrate normal coordinated walking movements after the exposure period were considered dead. Each test treatment consisted of two replicates of ten roaches each. The control roaches that were dipped in solutions containing 5 per cent acetone and 0.02 per cent emulsifier experienced 7 per cent mortality so data on the treated lots were corrected accordingly.

Flour beetle test. Adult confused flour beetles of variable age were randomized and exposed to treated filter papers in 9 cm. Petri dishes. The residues on the filter papers were obtained by evaporation of 2 ml. of an acetone solution of the toxicant. These data were based on six-day readings, using immobility as the criterion of death, and were corrected against a 3 per cent check mortality.

RESULTS

In view of the variation in results between individual tests, which in some cases did not provide a single finite value on the log-probit scale,

[2] Triton X-155, an alkyl phenoxy polyethoxy ethanol, Rohm & Haas Company, Philadelphia, Pa.

statistical analysis was not warranted. LD_{50} values based upon the criteria of death indicated above were estimated for each test from plots on log-probability paper and were averaged to obtain the values presented in Table I. The data demonstrate that the eta isomer of benzene hexachloride displays insecticidal activity equivalent to that of the alpha and delta isomers. From a practical standpoint the eta isomer of benzene hexachloride may be considered non-insecticidal.

TABLE I

INSECTICIDAL ACTIVITY OF FOUR ISOMERS OF BENZENE HEXACHLORIDE AND OTHER REPRESENTATIVE INSECTICIDES TO FOUR SPECIES OF INSECTS

Material tested	Average estimated LD50 values* in p.p.m. against			
	Musca domestica	*Blatella germanica*	*Aedes aegypti*	*Tribolium confusum*
Alpha BHC	>10,000	850	1,800	>10,000
Gamma BHC	1.2	3.0	0.64	6.1
Delta BHC	1,600	9,000	4.7	>10,000
Eta BHC	>10,000	4,400	34	9,200
BHC	3.3	13	2.3	245
DDT	6.5	205	0.009	54
Chlordane	1.7	7.0	3.5	17
Pyrethrins	66	40	0.08	175
TEPP	6.1	23	0.75	—

* Values for BHC, DDT, and chlordane were computed on the basis of technical grade products; other materials on a 100 per cent active ingredient basis.

SUMMARY

The results of tests against four species of insects with the newly discovered eta isomer of 1,2,3,4,5,6-hexachlorocyclohexane, with other isomers, and with representative insecticides, are presented in this report. From a practical standpoint the eta isomer may be considered non-insecticidal.

LITERATURE CITED

1. HALLER, H. L. Wartime development of insecticides. Indus. & Eng. Chem. **39**: 467–473. 1947.
2. KAUER, K. C., R. B. DUVALL, and F. N. ALQUIST. Epsilon isomer of 1,2,3,4,5,6-hexachlorocyclohexane. Indus. & Eng. Chem. **39**: 1335–1338. 1947.
3. KOLKA, A. J., H. D. ORLOFF, and M. E. GRIFFING. Two new isomers of BHC. Presented before the Amer. Chem. Soc., Organic Div. 121st Meeting, Buffalo, New York, March 24, 1952; to be published.
4. LORD, K. A. The contact toxicity of a number of D.D.T. analogues and of four isomers of benzene hexachloride to *Macrosiphoniella sanborni* and *Oryzaephilus surinamensis*. Ann. App. Biol. **35**: 505–526. 1948.
5. METCALF, ROBERT L. Relative toxicities of isomeric hexachlorocyclohexanes and related materials to thrips. Jour. Econ. Ent. **40**: 522–525. 1947.

6. SHERMAN, MARTIN. Relative toxicity of the isomers of benzene hexachloride to several insects. Jour. Econ. Ent. **41**: 575–583. 1948.
7. SLADE, R. E. The gamma-isomer of hexachlorocyclohexane (Gammexane). Chem. & Indus. (London) p. 314–319. 1945.

EFFECTS OF IRREGULAR SEX RATIOS ON GROWTH RESPONSE DATA FROM NUTRITIONAL ASSAYS UPON BLATTELLA GERMANICA (L.)[1]

JOHN D. HILCHEY AND ROBERT L. PATTON[2]

INTRODUCTION

The German cockroach, *Blattella germanica* (L.), like many other animals, shows sex differences in its growth response to diets used in nutritional studies (1, 3, 7, 8). Pettit (7) and House (4, 5, 6) have indicated that the average sex ratio of this species is close to unity. Therefore, it has been assumed by most workers in the field that sex ratios contributed no bias to their results. Woodruff (8) has suggested that data taken after the maturity of the roaches might be adjusted in order to minimize any possible bias due to variation in the sex ratio. However, to the authors' knowledge, no one has practiced such an adjustment.

In the course of nutritional assays upon *Blattella germanica* studies were made of the relation of sex to weight, the variation in the sex ratio of groups of cockroaches selected for assay, and the effects of these two factors upon the analysis of nutritional data. Data are presented in this report both to illustrate the false conclusions which may be drawn from data derived from small samples of roaches, and to demonstrate that by analyzing the data separately for the two sexes the effects of irregular sex ratios may be avoided.

EXPERIMENTAL PROCEDURE

The assays were carried out according to the methods described by House (4, 5, 6). Individual cockroaches were reared under aseptic conditions until death occurred or maturity was attained. Their weights determined at weekly intervals were used as the index of growth. In addition, the sex and the dates of hatching, moulting, and maturity were recorded for each insect. Tests of asepsis were performed on each individual so that those contaminated during the assay could be eliminated from further consideration.

Statistical analyses were carried out by the methods of Student's "t" test (2); comparisons were drawn between the control diet and one of the

[1] This research was conducted at Cornell University, Ithaca, New York, with the aid of a grant from the Lalor Foundation of Wilmington, Delaware.

[2] Entomologist, Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York, and Insect Physiologist, Department of Entomology, Cornell University, Ithaca, New York, respectively.

Copyright, 1952, by Boyce Thompson Institute for Plant Research, Inc.

test diets which illustrate both the effect of an irregular sex ratio upon analysis and the means by which this effect may be nullified.

Since the present experiment is concerned only with illustrating the effect of irregular sex ratios upon the analysis of nutritional data, the actual composition of the diets used is a secondary consideration. Therefore the statistical evaluations were limited to those diets which contribute to the understanding of the present problem.

RESULTS AND DISCUSSION

Data are presented in Table I illustrating the differences between the weights of male and female *B. germanica* during the first twelve weeks of life. Since none of these roaches were contaminated, the differences in rate of growth may be attributed to the greater capacity for growth which is characteristic of the female. It will be noted that there was a gradual in-

TABLE I

DIFFERENCES BETWEEN WEIGHTS OF MALE AND FEMALE BLATTELLA GERMANICA REARED ON THE CONTROL DIET

Age of cockroaches, weeks	Mean weight (in mg.) of cockroaches		Difference in weight, mg.
	Male	Female	
1	2.4±0.1	2.3±0.1	− 0.1
2	4.2±0.2	4.1±0.2	− 0.1
3	6.1±0.5	6.6±0.3	+ 0.5
4	7.8±0.6	8.5±0.5	+ 0.7
5	11.4±0.9	11.9±0.6	+ 0.5
6	14.6±1.3	16.9±0.6	+ 2.3*
7	18.4±1.3	19.3±1.5	+ 0.9
8	22.9±2.6	27.6±2.1	+ 4.7
9	32.4±2.9	36.5±2.7	+ 4.1
10	34.2±2.2	48.0±3.6	+13.8**
11	42.0±1.9	56.1±2.9	+14.1**
12	42.9±1.4	64.6±3.4	+21.7**

* Difference is significant at odds of 19:1.
** Difference is significant at odds of 99:1.

crease in the difference between the mean weekly weight of males and the mean weekly weight of females throughout the first nine weeks of assay. Differences first became highly significant in the tenth week of assay. The apparent significance of the difference in the sixth week may be classed as sampling variation.

Table II shows that the over-all sex ratio approximates unity; however, it may depart radically from this value within a group of roaches selected from several capsules for use in the evaluation of a single diet. Likewise, the sex ratio within the group of roaches comprising the "hatch" from a single egg capsule may vary within wide limits. Diet No. 1 and capsule "E" are excellent examples. Limitations are imposed by the number of cockroaches available from each capsule as well as by the number of diets

which must be run at the same time. Therefore each assay of a diet must be carried out with a small number of cockroaches selected from a relatively small number of capsules. Accidental deaths may increase the variation in the sex ratio. Multiple replications of diets would reduce the variation of the sex ratio, but the limitations of time, space, and cost prohibit the use of such a technique in this type of nutritional study.

The data in Table III demonstrate the biasing effect of an irregular sex ratio upon the results of a statistical analysis. It also demonstrates the re-

TABLE II

SEX DISTRIBUTION OF BLATELLA GERMANICA USED FOR NUTRITIONAL ASSAYS

As Hatched from Capsules

Sex	Capsule						
	A	B	C	D	E	F	Total
Males	13	16	12	13	8	10	72
Females	9	7	10	12	17	14	69
Ratio of males to females	1.44	2.29	1.20	1.08	0.47	0.71	0.96

As Distributed to Diet

Sex	Diet						
	Control	1	2	3	4	5	Total
Males	11	18	12	8	11	12	72
Females	14	7	11	12	13	12	69
Ratio of males to females	0.79	2.57	1.09	0.67	0.85	1.00	0.96

medial effect of a more critical evaluation of the data. When the data obtained from male and female cockroaches were analyzed collectively, the difference between the mean weights of insects fed different diets was found to be significant. This conclusion is shown to be in error when data derived from males and females are considered separately. The large proportion of males on diet No. 1 was sufficient to reduce the mean weight significantly. The data in Table III also show that the difference between the mean weights of the roaches fed on the control and No. 1 diets is not significant in the eighth week. This is due to the fact that the difference in the mean weights of the sexes did not become significant until after the eighth week of assay.

An irregular sex ratio increases the experimental error of a dietary assay beyond the level inherent in a nutritional trial. This increase in the experimental error may not be significant when the differences between the

weights of the animals fed the control diet and the weights of those fed test diets are sufficiently large. However, the sex ratio must be considered when differences are of small order only.

It has been suggested by some workers that in order to avoid the bias imposed by sex upon statistical comparisons, the data used should be taken only in a period before the time when sex differences become significant. Since the greatest portion of growth takes place during late nymphal and early adult life, the effects of nutritional deficiencies may not be reflected

TABLE III

STATISTICAL ANALYSIS OF THE WEIGHTS OF BLATTELLA GERMANICA REARED ASEPTICALLY

Diet	Datum	8th Week data	12th week data		
		Collective analysis	Collective analysis	Grouped males	Grouped females
Control diet	Mean weight in milligrams	25.5	55.1	42.9	64.6
	Standard error	1.7	2.9	1.1	3.4
	Number of insects	25	25	11	14
Diet No. I	Mean weight in milligrams	22.8	46.4	41.3	59.4
	Standard error	1.2	2.4	1.8	3.9
	Number of insects	25	25	18	7
Difference between means in milligrams		−2.7	−8.7*	−1.6	−5.2

* Difference is significant at odds of 19:1.

statistically in the growth until late in nymphal life. Only by comparing the performances of males and females separately may the bias due to sex be avoided while allowing adequate time for the proper assay of a diet.

It may be pointed out in Table III that although the standard error of the grouped males for the tenth, eleventh, and twelfth weeks of growth is *less* than that of the ungrouped data, the standard error of the grouped females is *greater* than that of the ungrouped data. Properly interpreted, the statistics of the data grouped according to sex emphasize: first, there are significantly different norms for the weights of male and female *B. germanica;* and second, the males range less in weight than do the females. It is clear that the data not grouped according to sex and which were obtained during either the ninth, tenth, eleventh, or twelfth week of assay are not homogeneous within diets. Therefore, the use of a single mean and standard error for each diet is misleading. A true picture of the homoge-

neity of such data may be presented only by the use of separate means and standard errors for male and female *B. germanica*.

SUMMARY

Female *Blattella germanica* (L.) were significantly heavier than males from the same population reared for twelve weeks under the same standard conditions. A positive trend first appeared during the third week and became highly significant in the tenth week. In dealing with aseptic nutritional assays upon *B. germanica* small test populations must be used; under these conditions sampling errors produced variations in the sex ratio of males to females of from 2.57 to 0.67. When data subject to these variations are analyzed without due consideration, erroneous conclusions may be drawn. The errors due to sampling may be avoided by evaluating the performances of males and females separately.

LITERATURE CITED

1. BOWERS, ROSS ELDON. The vitamin and lipid requirements of the cockroach, *Blattela germanica* L. Thesis (Ph.D.) Cornell Univ. 221 pp. 1940.
2. FISHER, R. A. Statistical methods for research workers. 5th ed. 319 pp. Oliver & Boyd, London. 1934.
3. HABER, VERNON RAYMOND. Some facts concerning cockroach biology, with special reference to the German cockroach, *Blatella germanica* Linn. Thesis (Ph.D.) Cornell Univ. 62 pp. 1924.
4. HOUSE, HOWARD L. Nutritional studies with *Blattella germanica* (L.) reared under aseptic conditions. II. A chemically defined diet. Canadian Ent. **81**: 105–112. 1949.
5. ———— Nutritional studies with *Blattella germanica* (L.) reared under aseptic conditions. III. Five essential amino acids. Canadian Ent. **81**: 133–139. 1949.
6. HOUSE, HOWARD L., and ROBERT L. PATTON. Nutritional studies with *Blattella germanica* (L.) reared under aseptic conditions. I. Equipment and technique. Canadian Ent. **81**: 94–100. 1949.
7. PETTIT, LINCOLN COLES. The roach, *Blattella germanica* (Linn.): its embryogeny, life history, and importance. Thesis (Ph.D.) Cornell Univ. 121 pp. 1940.
8. WOODRUFF, LAURENCE C. The cockroach, *Blattella germanica* Linn., as a test animal for nutritional studies. Thesis (Ph.D.) Cornell Univ. 90 pp. 1934.

AN ELECTRICAL MOISTURE METER FOR THE DETERMINATION OF MOISTURE IN COFFEE BEANS[1]

LELA V. BARTON AND A. GOENAGA

The consistent preparation of high quality green coffee (*Coffea arabica* L.) is a matter of vital concern to the coffee industry. According to Johnston and Foote (2) the process depends on the substitution of controlled pectic enzyme action for spontaneous fermentation, and substitution of rapid drying for slow and inefficient drying. The method of drying and the amount of moisture present in the seeds is also of importance in their retention of viability in storage (4).

Perhaps the most extensive research on coffee is being done at the present time in Brazil at the Instituto Agronomico de Campinas. A detailed description of this work together with a bibliography has been published by Ramirez Bermudez (3), who gives the different drying methods currently in use. Formerly all coffee was dried in drying yards and required from 10 days to 3 weeks and much labor. Now artificial drying systems are being introduced, shortening the drying time to a few hours. The combination of the shortened time and the higher temperatures for drying makes the recognition of the end point of drying much more critical. No information is available, however, on methods of determining the actual moisture content of the coffee berries or seeds. A standard method, especially one which could be used easily under field conditions as well as in the laboratory for determination of the moisture present in fruits or seeds, would be a big step forward in producing a commercial product of uniform quality. It was thought that the Universal Electric Moisture Tester might meet these requirements.

Consequently an experiment was designed to test the reliability of this instrument. The data obtained showed that when the moisture content of coffee in "coco" is within the range of 10 to 23 per cent of the dry weight of the coffee, the Universal Moisture Tester may be used satisfactorily.

MATERIALS AND METHODS

The coffee in "coco" used in these tests was shipped by air express from São Paulo, Brazil, through the courtesy of Dr. F. P. Mehrlich of International Basic Economy Corporation, and was received in this laboratory on September 13, 1951. It should be pointed out that coffee in "coco" is a

[1] This experiment was sponsored by the Burrows Equipment Co., Evanston, Illinois, and Sheldrick Manufacturing Co., Upper Sandusky, Ohio.

Copyright, 1952, by Boyce Thompson Institute for Plant Research, Inc.

heterogeneous mixture of whole fruits, broken fruits, clean seeds and fruit coats, with whole fruits comprising most of the mixture. The mixture was used as received since it is this form which is most often marketed and for which moisture determinations are desired. The non-uniformity of the material undoubtedly increased the sampling error, but results were fairly consistent as will be seen from the data presented below. The seeds were of the Red Bourbon variety.

The first step in the procedure was to adjust the moisture content of various samples, so that they represented a range comparable to that which might be of interest to the coffee industry. This was done at first by placing samples at laboratory temperature in desiccators over different solutions giving a range in relative humidity. However, this method did not prove practical for coffee in "coco" since mold appeared before the time necessary for equilibrium moisture adjustment had elapsed.

Successful moisture adjustment was secured by placing weighed lots of 400 grams each in a 5° C. room, the atmosphere of which was saturated with moisture. A previous moisture determination in a vacuum drying oven of the source lot for the samples had shown about 12 per cent moisture. From this figure, then, it was easy to calculate the approximate desired weights of the 400-gram samples. For example, an increase to 408 grams would indicate the attainment of about 14 per cent moisture content. In other samples, higher moisture contents could be secured.

For moisture contents below 12 per cent, drying was necessary. Again, 400-gram lots were weighed and dried over calcium oxide in a desiccator or in a vacuum oven at 73° C. to the approximate desired weight.

In this way a range of moisture contents from about 5 to about 35 per cent was secured. The desired weights were obtained after a few days, or, in some cases, a few hours in the moist room or the dry desiccator or the vacuum oven. Thus, the fruits did not have time to mold.

Immediately after the seed lot had attained the desired weight, it was placed in a bottle large enough for the lot, but with little extra space. The bottle was fitted with a screw cap, which was sealed with paraffin. The sealed bottles were then placed at 0.5° C. where they were allowed to remain for from 25 to 49 days so that the moisture in the seed would be evenly distributed throughout the lots before actual moisture contents were determined.

Before samples were taken for moisture determinations, the bottles were removed from the low-temperature chamber and allowed to come to laboratory temperature. No mold appeared on any of these lots at any time during the experimental period.

Since coffee fruits contain a large percentage of oil, the procedure for moisture determination for a standard was not known. Tests were made on 17 different lots of seeds, where duplicate samples were dried for 48

hours in a vacuum oven at 73° C., and other duplicate samples for 22 hours in a vacuum oven at 100° C. A limited number of samples were also dried in a forced draft oven at 47° C. for 42 and 66 hours. The last method removed only a small percentage of the moisture believed to be in the fruits. Data for the other two methods will be presented. It is believed that the drying in a vacuum oven at 100° C. gave a more accurate moisture determination than the lower temperature, since there was no evidence of "roasting" and the moisture percentages were somewhat higher than for the lower temperature. Also a vacuum oven at 105° C. has been found satisfactory for moisture determination of oily soybean seeds (1). Additional work would have to be done to establish the best standard procedure for the determination of the moisture content of coffee fruits and seeds, but it is believed that the present results are of sufficient accuracy to be significant. For vacuum-oven moisture determinations, lots of seeds of approximately 8 to 30 grams, depending upon the size of the weighing bottle, were weighed to the fourth place in grams on an analytical balance before and after drying. From these weights, the weight of water and the percentage moisture based on both wet and dry weights of the seeds were calculated.

At the end of the storage period at 0.5° C., samples of "coco" lots were taken for vacuum-oven determination of moisture, at the same time that 35-gram samples of the same lots were taken for testing in the Universal Moisture Tester. At a later time, measured samples of the same lots which had been resealed and replaced at 0.5° C. were tested in the Universal Moisture Tester. The volumetric measuring cup (85 ml.) for large grains supplied by Burrows Equipment Company was used.

In the Universal Moisture Tester, the electrical component records the electrical resistance of a sample of known weight or volume compressed to a given thickness. This resistance varies with the moisture content of the sample and with the temperature. The temperature is recorded on a thermometer which has been built in the unit. A simple correlating dial allows compensation for temperature which is lined up with the meter reading after which the moisture per cent may be read directly on a third scale. No outside source of electrical supply is required since the instrument has a generator which is cranked by hand. This permits its use in both laboratory and field.

RESULTS

Moisture contents of 8 per cent or less could not be measured accurately using the Universal Moisture Tester. Results of tests with lots of higher moisture contents are shown in Tables I and II and in Figure 1.

In Table I measurements are given for some vacuum-oven moisture determinations and for samples used in the Universal Moisture Tester.

TABLE I

PER CENT MOISTURE IN DUPLICATE SAMPLES OF COFFEE IN "COCO" ADJUSTED TO DIFFERENT MOISTURE CONTENTS

Vacuum oven, dry wt. basis		Universal Moisture Tester						
		35-Gram samples						85 Ml. sample
73° C.	100° C.	0.700*		0.600*		0.500*		0.500*
		0 Min.	5 Min.	0 Min.	5 Min.	0 Min.	5 Min.	5 Min.
7.95	10.16	10.85	11.00	11.60	11.70	12.15	12.20	11.95
8.07	10.12	10.90	10.95	11.45	11.60	12.15	12.20	11.85
8.95	11.05	12.55	12.62	13.10	13.12	13.55	13.55	12.85
8.92	10.91	12.65	12.79	13.19	13.20	13.60	13.61	12.50
12.07	14.24	15.45	15.50	15.97	15.97	16.70	16.75	15.75
12.55	14.01	14.65	14.70	15.20	15.25	15.75	15.77	15.90
10.25	—	12.80	12.90	13.40	13.50	13.92	13.92	—
10.17	—	12.45	12.55	13.05	13.10	13.85	13.85	—
12.97	14.67	15.70	15.75	16.60	16.60	17.40	17.40	16.40
13.16	15.23	15.35	15.40	16.20	16.20	17.10	17.10	17.00
14.70	17.10	17.80	17.90	18.75	18.75	19.89	19.89	19.50
14.60	16.85	17.35	17.40	18.10	18.10	19.35	19.40	18.50
14.84	18.45	18.10	17.90	18.90	18.95	19.90	19.90	19.95
15.39	17.34	18.42	18.55	19.50	19.50	20.30	20.30	19.20
16.56	19.86	18.00	17.90	18.70	18.70	19.75	19.80	20.90
16.74	18.88	19.40	19.22	20.20	20.20	21.10	21.20	20.90
18.94	20.45	19.15	19.30	20.50	20.54	21.50	21.55	22.00
17.77	20.23	19.70	19.90	20.82	20.82	22.20	22.18	22.00
19.34	20.63	19.80	19.90	21.00	21.00	22.19	22.19	23.10
19.29	21.13	21.40	21.40	22.50	22.50	24.00	24.00	22.60
21.42	23.76	22.30	22.40	23.60	23.60	25.19	25.19	24.60
21.31	23.62	22.10	22.10	23.30	23.30	25.10	25.10	26.40
21.29	23.30	22.60	22.70	23.80	23.80	25.59	25.59	26.20
21.72	23.91	22.60	22.60	24.20	24.22	25.80	25.50	25.75
26.10	26.56	26.98	27.22	30.22	30.22	34.10	34.10	—
25.46	27.37	26.50	26.42	28.50	28.80	32.25	32.25	—
36.77	39.64	34.20	34.90	40.20**	41.00**	45.00**	45.00**	Above
36.41	35.54	37.90	39.60	45.00**	45.50**	50.00**	50.00**	40.00

* Compression in inches.
** Approximate.

The precision of the two methods is indicated by the agreement between duplicate samples.

It will be noted that slight compression of 35-gram samples (to 0.700 inch) gave a close approximation of the actual moisture content as meas-

ured in a vacuum oven at 100° C. but the differences between the values obtained by the two methods were not the same for all moisture content levels. The same characteristic was noted for moisture contents obtained after compression to 0.600 inch. With further compression to 0.500 inch, however, consistent differences between vacuum-oven and electrical

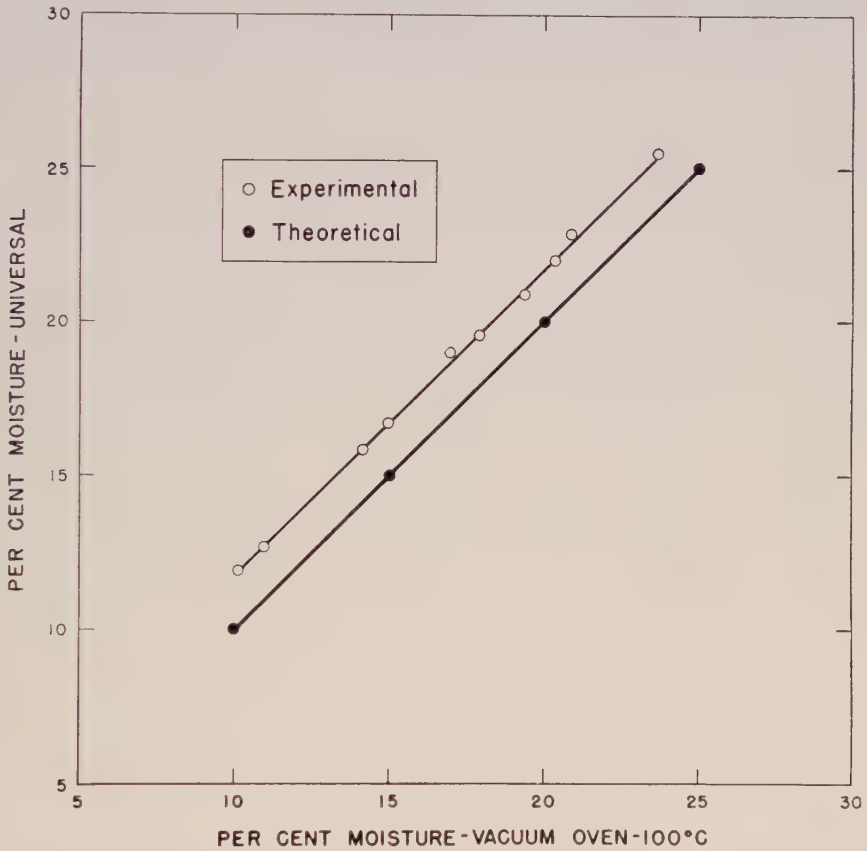


FIGURE 1. Calibration of Universal Moisture Tester for moisture determination of coffee in "coco" by comparison with moisture determinations made by drying in a vacuum oven at 100° C.

moisture determinations were secured over a wide range of moisture contents. Two readings were made on the Universal Moisture Tester after each compression, one immediately after compression, and the other after a five-minute interval to allow for possible temperature adjustment. As will be seen, there are no significant differences in these readings. However, for the establishment of a calibration curve it seemed desirable to use the five-minute readings.

Samples measured in the volumetric measuring cup supplied by Burrows Equipment Company were more uniform in behavior than weighed lots. This is more easily seen in Table II. These figures are averages of duplicate tests. All readings on the Universal Moisture Tester were taken five minutes after compression to 0.500 inch. Differences between moisture percentage readings on the Universal Moisture Tester and those obtained by drying the fruits in a vacuum oven, show the advantage of the 100° C. oven over the 73° C. oven and of measured over weighed lots. As compared

TABLE II

COMPARISON OF WEIGHED AND MEASURED LOTS OF COFFEE IN "COCO" FOR DETERMINING MOISTURE CONTENT IN THE UNIVERSAL MOISTURE TESTER AS MEASURED BY MOISTURE DETERMINATIONS OBTAINED BY DRYING IN A VACUUM OVEN AT 73° OR 100° C.

Universal Moisture Tester (%)		73° C. vac. oven			100° C. vac. oven		
35 G.	85 Ml.	% Dry weight	Difference*		% Dry weight	Difference*	
			35 G.	85 Ml.		35 G.	85 Ml.
12.20	11.90	8.01	4.19	3.89	10.14	2.06	1.76
13.58	12.68	8.94	4.64	3.74	10.98	2.60	1.70
16.26	15.83	12.31	3.95	3.52	14.13	2.13	1.70
17.25	16.70	13.07	4.18	3.63	14.95	2.30	1.75
19.65	19.00	14.65	5.00	4.35	16.98	2.67	2.02
20.10	19.58	15.12	4.98	4.46	17.90	2.20	1.68
20.50	20.90	16.65	3.85	4.25	19.37	1.13	1.53
21.87	22.00	18.36	3.51	3.64	20.34	1.53	1.66
23.10	22.85	19.32	3.78	3.53	20.88	2.22	1.97
25.15	25.50	21.37	3.78	4.13	23.69	1.46	1.81
25.55	25.98	21.51	4.04	4.47	23.61	1.94	2.37
33.18	—	25.78	7.40	—	26.97	6.21	—
47.50**	—	36.59	—	—	37.59	—	—

* Difference between moisture percentages as shown for determinations in vacuum oven and Universal Moisture Tester.

** Approximate.

Note: Recommendation for use of Universal Moisture Tester. Compress volumetric sample to 0.500 inch and subtract 1.75 per cent from the final reading to arrive at the moisture content as determined in a vacuum oven at 100° C., e. g., 11.90 − 1.75 = 10.15.

with determinations in a vacuum oven at 73° C. the average differences, excluding the two highest moisture contents, were 4.17 for the weighed and 3.96 for the measured lots. Corresponding figures resulting from a comparison with percentages obtained from drying in a vacuum oven at 100° C. were 2.02 and 1.81. Averages of duplicates from 100° C. oven and measured lots were used to plot the experimental line (open circles) shown in Figure 1. The nature of the line obtained, as compared with the heavier line which represents the ideal, i.e., complete agreement of values obtained by the two methods, would seem to justify the use of a single

compression and a single correction (−1.75 per cent) for the determination of moisture content of coffee in "coco" with moisture percentages ranging from 10 to 23 per cent of the dry weight of the fruits. This range is within the practical need for moisture determination. This fact coupled with the desirability of using measured instead of weighed samples simplifies the procedure so that readings could be made quickly and accurately with no special equipment and no special training of the operator.

To determine what part of the fruit retains most moisture, fruit coats were removed from some of the seeds held in the laboratory. It is believed that very little of the moisture was lost in the process of coat removal. Vacuum-oven drying at 73° C. gave duplicate moisture percentages as shown in Table III.

TABLE III

MOISTURE DETERMINATION OF DIFFERENT PARTS OF THE COFFEE FRUIT

Material used	Moisture (%)	
	Wet wt. basis	Dry wt. basis
Intact fruits	11.15 11.20	12.55 12.62
Fruit coats	11.68 11.91	13.23 13.52
Seeds	10.51 10.51	11.75 11.75

It is seen that the fruit coats of this particular lot hold very little more moisture than the seeds themselves. This fact emphasizes the probable uniform moisture distribution in the samples used in the above tests.

Limited tests on clean coffee seeds (free of the berry) indicate that the Universal Moisture Tester may be used for determination of their moisture content though a different calibration curve would be necessary.

CONCLUSION

The determination of the moisture content of coffee in "coco," when that moisture is within the range of 10 to 23 per cent of the dry weight of the coffee, may be made on the Universal Moisture Tester using measured lots (standard volumetric cup for large grains) compressed to 0.500 inch, and applying a small correction (−1.75 per cent). This recommendation can be made even though coffee in "coco" is made up of a heterogeneous mixture of whole fruits, broken fruits, clean seeds and fruit coats.

LITERATURE CITED

1. BECKEL, A. C., and F. R. EARLE. A study of moisture in soybeans. Indus. & Eng. Chem. Anal. Ed. **13**: 40–43. 1941.
2. JOHNSTON, W. R., and H. E. FOOTE. Development of a new process for curing coffee. Food Technol. **5**: 464–467. 1951.
3. RAMIREZ BERMUDEZ, JOSE. El instituto agronomico de campinas y la fitotecnia aplicada en el mejoramiento del cafe. Departamento de Enseñanza y Divulgacion Agricola. Ministerio de Agricultura. Guatemala, C. A. 1951. 133 pp.
4. Storing coffee seed. Trop. Agric. [Trinidad] **18**: 25. 1941.

GERMINATION AND CHEMICAL CONTROL OF THE GIANT FOXTAIL GRASS

LAWRENCE J. KING[1]

INTRODUCTION

Within the space of two decades the aggressive giant foxtail grass (*Setaria Faberii* Herrm.) has been introduced into this country, has appeared in a large number of states in the east and in the middle west, and has become established in the cornbelt area as a very serious competitive weed in the corn and soybean crops. Attempts to control this weed by several available chemicals have raised a number of problems relative to its germination and seedling development, which, if clarified, might provide the basis for more effective control measures. Consequently certain of these studies have been initiated under the controlled conditions of the laboratory and greenhouse.

This annual weed is believed to have been introduced into this country as a contaminant in large lots of Chinese millet (38). The seed was first detected by a seed analyst in 1932 (7), and the first plants were found in 1936 near a greenhouse at Arlington Farm, Virginia (1). Other records of its appearance have come from Illinois, Iowa, Indiana, and Missouri, and from several eastern seaboard states (13, 16, 40, 41, 44, 51). In the cornbelt area this grass is especially abundant in corn and soybean plantings, developing rapidly following the last cultivations.

Setaria Faberii is a member of the foxtail millets, and in seed structure and growth habits is closely related to *Setaria viridis* (L.) Beauv. (24, 28, 38, 47). It is chiefly distinguishable from the latter in that the plant is considerably larger, the leaves are pubescent, and the panicle is conspicuously nodding (19, 25, 26, 40), thus providing the common name, giant foxtail, or nodding foxtail (Fig. 1). Its size is related undoubtedly to the fact that it is a tetraploid (33). Specimens over six feet in height have been observed, especially in rich bottomland cornfields. It apparently is not able to compete with other species in ground which is uncultivated, becoming then dwarf and finally disappearing altogether. It grows best in open waste ground recently abandoned from cultivation and in such areas may become a much branched sprawling plant, three feet in height. In poor sod ground, where *Setaria lutescens* (Weigl) Hubb. and *S. viridis* are quite at home, *S. Faberii* is less adaptable and becomes of small size since it cannot compete with the

[1] Senior Fellow in Plant Physiology, Boyce Thompson Institute for Plant Research, Inc., Yonkers 3, N. Y., and the Carbide and Carbon Chemicals Co., New York 17, N. Y. The author is greatly indebted to Mr. Herbert Hatfield Jr. for valuable assistance in conducting these experiments.

native grasses (1). Depauperate flowering forms only three inches in height have been reported for this species (43), as well as for other species of *Setaria* (2).

Giant foxtail grass appears to germinate all through the season well into the fall. It is a short-day plant, similar in this respect to *S. italica* (15).

It has been found in this laboratory that seed of giant foxtail grass not

FIGURE 1. Single plant of giant foxtail grown in the greenhouse; about 60 days from seed. (Photographed on July 7, 1952.)

subjected to outdoor winter conditions germinated in erratic fashion. Generally higher germination occurred in soil than on moist filter papers, and also higher following moist storage at 21° C. Alternate wetting and drying of soil or filter paper cultures stimulated greater germination. The presence of inhibitory substances in the seeds was indicated, and their role in dormancy and periodic germination are considered. Depth of planting experiments and soil compaction provided data bearing on the possible effectiveness of preemergence chemical applications to the soil surface. Several weed control chemicals were effective in controlling the nondormant seeds, but dormant seeds were not destroyed.

MATERIALS AND METHODS

The seed for these studies was all from one lot collected in the fall of 1951 near Urbana, Illinois, by Professor F. W. Slife of the University of

Illinois, and sent for study by Mr. J. E. Edwards. The seeds were stored in the laboratory at room temperature, and were utilized as received, the glumes not being removed. A cutting test on a lot of 200 seeds revealed about 20 per cent to be imperfect or unfilled. Thus the maximum germination possible would be about 80 per cent. Other details of procedure are given under the appropriate sections.

RESULTS AND DISCUSSION

GERMINATION

Coat effects. Erratic germination of the seeds of giant foxtail under controlled conditions was noted from the first test made. To determine whether the fruit coats might account for some of these effects, coats were removed before planting in soil in 4-inch clay pots in the greenhouse. A second group of seeds was scarified by nicking the seed coat with a file and then planted in pots. Results of one such experiment showed only 4 per cent germination in the seeds which had the fruit coat removed and no germination in the seeds which were scarified after fruit coat removal, while the control gave one of the highest germination values recorded, 82 per cent.

It would appear from the above data that seeds with the fruit coat removed are susceptible to embryo injury when planted in the soil, possibly by the entry of soil pathogens. Some injury may have been caused to the embryos during the scarification process. At any rate, the fruit coat does not appear to be an important factor in the prevention of germination of giant foxtail seeds.

Germination medium. Attempts to germinate the seeds of giant foxtail on filter paper in Petri dishes resulted in small germination percentages, usually not more than 34 per cent (Table I). The effect of adding soil to the filter paper was studied. Seeds were cultured on moist filter papers, with and without soil, in 15 cm. Petri dishes in the dark at 21° C. and in the daylight at room temperature. The results showed an increase of about 10 per cent both in darkness and in light with the addition of soil, but germination values were still quite low. Values taken at 21 days gave for the dark series 32 per cent with soil and 23 per cent without soil; while in the light series values of 43 per cent for the soil culture, and 31 per cent for those without soil were recorded.

A series of tests was conducted to determine whether the beneficial effect of soil addition may have been due to the soluble nutrients. Seeds were placed in 30 ml. beakers to which 1 ml. of solutions of potassium nitrate (at 0.5, 1.0, 2.0 per cent), sodium thiocyanate (at 0.01, 0.1, and 1.0 per cent), or sodium metaarsenite (at 10.0 per cent) was added. Twenty-five seeds were used in each of two replicates. Seeds were soaked for 24 hours after which they were removed and rinsed several times to remove any

traces of the chemical from the seed coat. The seeds were planted in soil in 4-inch clay pots and allowed to germinate in the greenhouse. The results are shown in Table I. Increases in germination were secured from seeds soaked in a 2.0 per cent potassium nitrate solution or a 1.0 per cent sodium thiocyanate solution. Apparently the amount of the sodium arsenite that was absorbed by the seeds was sufficient to kill the embryo.

Temperature effects. Temperature fluctuation in soil plantings as well as the nutrients present may account for some of the increased germination of seeds in pots over those kept at constant temperature in Petri dishes. Seeds were cultured on moist filter papers at alternating temperatures of 21° to 30° C. and 21° to 37° C. They were kept in the 21° C. room for 16

TABLE I

GERMINATION IN SOIL OF GIANT FOXTAIL SEEDS

Days after planting	Average per cent germination of 50 seeds soaked in the various chemicals at the concentrations indicated								
	$NaAsO_2$ (%)	KNO_3 (%)			NaSCN (%)			H_2O	Dry
	10.0	2.0	1.0	0.5	1.0	0.1	0.01		
7	0	44	12	18	36	6	22	20	28
14	0	54	22	34	44	8	34	28	30
21	0	60	30	34	54	18	38	28	34

hours and at the higher temperatures of 30° and 37° C. for eight hours each day. This treatment was continued for four consecutive 24-hour periods after which the seeds were allowed to remain at 21° C. for the remainder of the experiment. Controls were also set up at constant temperatures of 21°, 30°, and 37° C. Duplicates of 50 seeds each were used. Results taken after 14 days showed a maximum germination of 48 per cent for the 21° to 37° C. alternation, in contrast to 10 and 20 per cent for the constant temperatures of 21° and 37° C. respectively. Many workers (9, 48, 50) have observed the beneficial effect of alternating temperatures on weed seed germination.

Another method commonly used to break seed dormancy is pretreatment on a moist medium at controlled temperature. Giant foxtail seeds were placed on moist filter paper at 5°, 10°, and 21° C. for seven days after which they were germinated in soil in the greenhouse. Seeds pretreated at 21° C. gave twice as much germination as the control, while the lower temperatures were less effective. Germination studies of another grass, *Panicum anceps* Michx., have disclosed that the dormancy could be overcome by moist storage for eight weeks at 5° C. (17). Periods longer than seven days were not tried in the present experiment.

Depth of planting. The effectiveness of chemical soil treatment for weed control quite frequently depends upon the depth at which the preva-

lent weeds germinate. Certain more deeply germinating weeds such as ragweed (*Ambrosia trifida* L.) may occasionally not be checked by the presence of chemicals on the soil surface. On the other hand, such shallow germinating weeds as crabgrass (*Digitaria* spp.), purslane (*Portulaca oleracea* L.), and chickweed (*Stellaria media* [L.] Cyrill.) are quite readily controlled. Contrary to the importance of knowing the optimum depth of planting for maximum germination of crop seeds, the maximum depth of weeds for even a minimum germination is quite important—not only for possible control of such weeds, but also for dissemination of the species. Oftentimes only one seedling need emerge and produce seed to maintain the weed as a pernicious one.

Hanf (23) divides the emergence process of weeds into two components —the germination itself, and the subsequent growth of the seedling in the soil. He states that the greater the seed weight, the greater is the ability to grow through the soil. He found the type of soil important, too, for in loose sandy soil emergence is possible with seeds at a greater depth than in solid clay soil.

In Table II are presented some data on seed weight and optimum and maximum depths of germination and seedling emergence that were compiled from the literature, or determined by the writer. From the data presented there is a reasonably valid correlation between seed weight and maximum depth of emergence though undoubtedly more data should be assembled. It is true that the rule-of-thumb method of planting seeds no more deeply than four times the diameter has long been the practice.

Depth of germination has been studied extensively in the wild oat, *Avena fatua* (27, 32, 48). Kirk and Pavlychenko (32) found that seedlings of the wild oat reached the surface of the soil from depths down to 7 inches (17.5 cm.), but that the length of the mesocotyl varied with the depth of sowing and the coleoptile node was always within 1 inch of the surface.

Waldron (49) observed that in the first year after planting *Setaria viridis* under field conditions, the optimum depth for emergence was 2.5 cm. with the maximum depth at 7.5 cm. Seeds planted at depths of 17.5 and 25 cm. did not emerge at any time during the five years of observation, but the seed removed from these levels after this period of time were found upon testing to be viable.

In the first experiment 25 seeds of giant foxtail were planted in each of two replicate rows at depths of 0, 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 cm. in shallow wooden greenhouse flats. The seeds were planted on January 10, 1952, and kept in the greenhouse at about 24° C. Germination counts were taken at weekly intervals up to 21 days. The results presented in Table III, Series A, show that the best germination occurred at the 0.5, 1.0, and 1.5 cm. depth with germination percentages of 34, 30, and 36 respectively after

TABLE II

COMPILED DATA ON THE 1000-SEED WEIGHT AND ON THE OPTIMUM AND MAXIMUM DEPTHS OF EMERGENCE IN SOIL FOR A NUMBER OF COMMON WEEDS*

Species name	1000-Seed wt. in g.	Optimum depth of emergence in cm.	Max. depth of emergence in cm.
Agrostemma githago L.	9.845††	0.5	10.0**
Ambrosia artemisiifolia L.	3.950	—	—
Ambrosia trifida L.	17.400	5.0	12.5 (49)
Anthemis cotula L.	0.240 (39)	—	—
Anthemis tinctoria L.	—	1.0	2.0**
Avena fatua L.	17.520	2.5	17.5 (32)
Avena fatua L.	—	—	12.5 (49)
Capsella bursa pastoris (L.) Medic.	0.096	0.5	2.0**
Centaurea cyanis L.	4.800†	0.5	8.0**
Chenopodium album L.	0.650 (42)	0.5–1.0	5.0** (35)
Cirsium arvense (L.) Scop.	1.575	1.0	6.0**
Digitaria sanguinalis (L.) Scop.	0.270 (39)	1.0†	4.0**
Galium boreale L.	0.600	—	—
Galium mollugo L.	—	1.5	4.0**
Matricaria inodora L.	—	0.5	2.0**
Matricaria matricariodes (Less.) Porter	0.130	—	—
Portulaca oleracea L.	0.130	—	—
Setaria Faberii Herrm.	1.727	0.5–1.5	3.0
Setaria Faberii Herrm.	—	—	12.0
Setaria glauca (L.) Beauv.	4.200 (39)	—	—
Setaria glauca (L.) Beauv.	1.280	—	—
Setaria italica (L.) Beauv.	1.830 (39)	—	—
Setaria viridis (L.) Beauv.	1.475	2.5	7.5 (49)
Sinapis (*Brassica*) *arvensis* L.	1.900	1.0	6.0
Sinapis (*Brassica*) *arvensis* L.	—	2.5	7.5 (49)
Stellaria media (L.) Cyrillo	0.505 (42)	1.0	2.0**
Thlaspi arvense L.	0.785	0.5	2.0**
Thlaspi arvense L.	—	—	5.0 (49)

* Unless otherwise noted the seed weights are from Stevens (46), and the depths of emergence from Kolk (34). The numbers in parentheses refer to the literature cited.

** Maximum depths tested.

† Determined by the author.

†† Data from D. Isleib.

21 days. The surface plantings germinated at 10 per cent. Only slight emergence occurred at 2.0 cm. (12 per cent) and at 3 cm. (4 per cent), while none emerged at 4.0 cm. at this time.

A test was devised to determine whether these seeds would emerge at a greater depth than 4 cm. Seeds were planted in 4-inch clay pots at depths of 1.0, 2.0, 4.0, 6.0, 8.0, and 9.5 cm. Two replicates each of 25 seeds were planted, and germination data were taken at 7, 14, and 21 days (Table III, Series B). Seedlings appeared on the fourteenth day from all planting depths—even at 9.5 cm. where there was 8 per cent germination. This was in striking contrast to the planting just described above where no seedlings emerged from a 4 cm. depth. One of these seedlings was removed and is shown in Figure 2 A.

It is true that the depth of 9 cm. in a 4-inch clay pot does not correspond to a similar depth under field conditions as Thurston (48) also admits. There is greater aeration both through the wall of the pot and through the opening on the bottom, as well as little compaction of the soil. Hence a wooden box 30×30 cm. and 25 cm. in depth was filled with greenhouse soil to a depth of 23 cm. Fifty seeds were planted in the center of the soil mass at a depth of 11.5 cm. After 30 days no seedlings had emerged, and

TABLE III

EFFECT OF DEPTH OF PLANTING AND SOIL DESICCATION UPON THE GERMINATION OF GIANT FOXTAIL SEEDS PLANTED IN GREENHOUSE FLATS (SERIES A) AND IN 4-INCH CLAY POTS (SERIES B)

Series	Depth of planting, in cm.	Average per cent germination of 50 seeds after days indicated				
		7	14	21	56*	56**
A	0	8	11	10	8	18
	0.5	0	34	34	6	40
	1.0	10	24	30	12	42
	1.5	8	20	36	8	34
	2.0	0	10	12	12	24
	3.0	4	4	4	8	12
	4.0	0	0	0	8	8
B	1.0	16	32	32	—	—
	2.0	8	22	22	—	—
	4.0	0	22	24	—	—
	6.0	0	16	20	—	—
	8.0	0	26	30	—	—
	9.5	0	8	8	—	—

* After 21 days the flats in Series A were permitted to dry out for an additional 21 days. Germination values given here are for the 14-day period following rewatering of soil.
** Total germination for the entire 56-day period.

at this time the top layer of soil was removed disclosing that three of the seeds had germinated, with one seedling having a shoot length of about 1.5 cm. This same block of soil was then again planted with seeds at depths of 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 cm. Results after 14 days gave emergence values of 18, 9, 9, 6, 12, 3, and 6 per cent respectively for the depths just mentioned. The first test was conducted during the period April 11 to May 11, 1952, and the second one during May 26 to June 9, 1952. It is quite possible that the soil was not as firmly packed in the second series as it was in the first and, too, the seeds were planted in rows with some of the seeds near the walls of the box.

The experiments involving depth of planting show the variability of results, and point out the need for standardizing the test methods. Determinations under actual field conditions would undoubtedly be the most useful. The novel methods of Dr. Martin Hanf (21, 22, 23) offer a new ap-

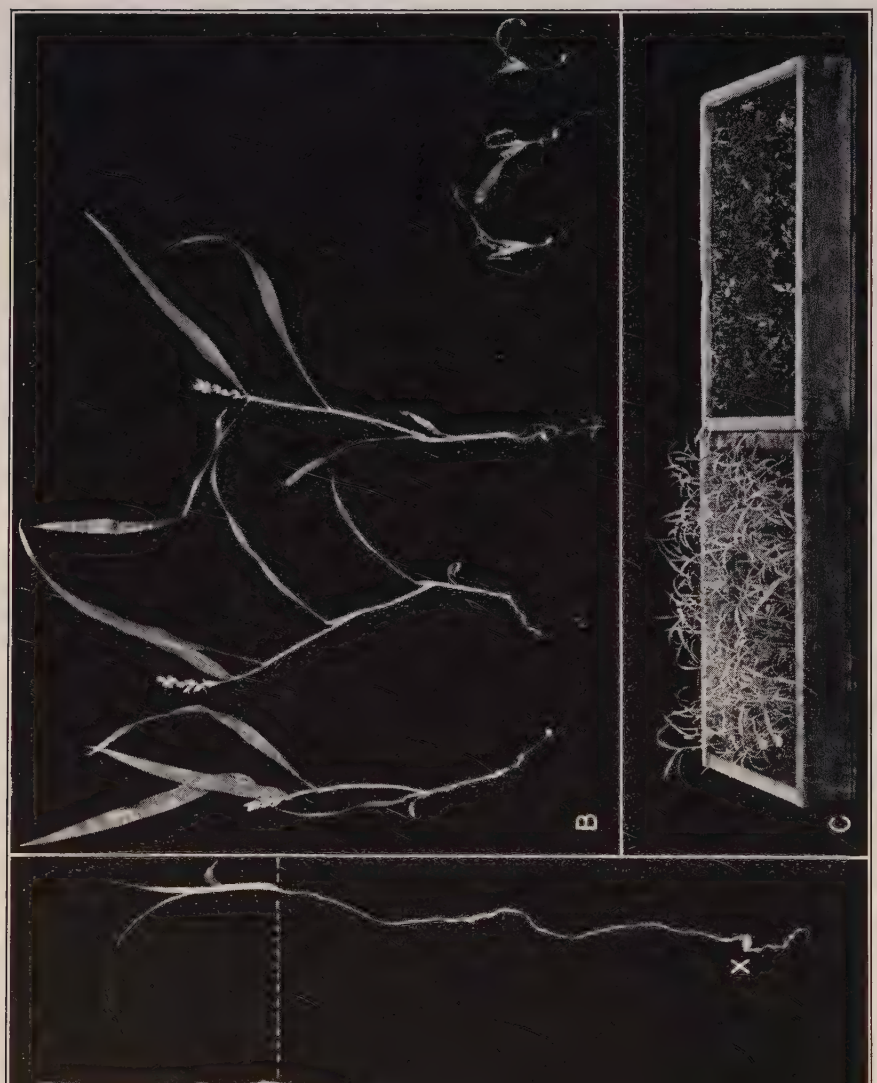


FIGURE 2. A. Seedlings of giant foxtail planted in 4-inch clay pots: (left) at normal depth of about 1.0 cm., and (right) at a depth of 9.5 cm. showing the effect of deep planting on the elongation of the mesocotyl. The dotted line marks the soil level, and the seeds are marked by an X. B and C. Control of giant foxtail grass by dichloral urea. B. Seedlings removed from the flats. (Left) Seedlings from the control flat showing normal growth and flower development during the short days of November 23 to December 27, 1951; (right) seedlings that are dwarfed and dying from the dichloral urea treated flat. C. (Left) Control flat; (right) flat showing control of seedling development following application of dichloral urea at

proach to this difficult problem. His method (22) of applying known weights to the soil surface offers a more exact method of studing soil compaction in relation to germination and seedling emergence. As shown by Figure 2 A, the mesocotyl grows to a great length if the seed is planted deeply. A mesocotyl length of 28 cm. has been recorded for Teosinte, and 36 cm. for a variety of maize cultivated since ancient times by the American Indians in regions where the drought is too severe for strains which need the ordinary shallow planting (2). Elongation of the mesocotyl has been recorded for timothy seedlings by Evans (11) who found that no matter how deeply the seed was planted the mesocotyl elongated so that adventitious roots were formed near the soil surface. Hanf (21) observed that in plants of *Galium aparine* from seeds sown in deeper layers of the soil (10 cm.) the main root system can be equalized by the formation of adventitious roots from the greatly elongated hypocotyl. Thus some dicotyledonous plants have a structure similar to the mesocotyl of certain monocotyledons for regulating the depth of the root zone.

It was also of interest here to determine whether several varieties of cultivated millet (*Setaria italica*) might have the capacity to germinate and emerge from a deep planting. Data for two varieties and the giant foxtail given in Table IV show that Early Fortune millet germinated quite well from a depth of 9 cm. while only 10 per cent of the giant foxtail seeds germinated from this depth. Western Common millet gave only 2 per cent at 9 cm. after 14 days, the poorest of all. It can readily be seen from the high germination values at 1 cm. that the cultivated millets exhibit no dormancy in contrast to the low values for the dormant giant foxtail.

TABLE IV

EFFECT OF THE DEPTH OF PLANTING UPON THE GERMINATION OF SEVERAL CULTIVATED VARIETIES OF MILLET SEED

No. of days after planting	Average per cent germination of 50 seeds at depth indicated					
	Western Common millet		Early Fortune millet		Giant foxtail	
	1 cm.	9 cm.	1 cm.	9 cm.	1 cm.	9 cm.
7	74	0	78	52	22	0
14	74	2	78	52	32	10

Effects of alternate wetting and drying. Some flats of soil from which only a few seedlings had emerged were allowed to dry thoroughly for a period of three weeks. At the end of this period water again was applied as in the earlier experiments. No disturbance of the soil had occurred, since the original seedlings were cut off at the soil level. After 14 days, counts were again made (Table III) when it was noted that seedlings occurred at all

depths of planting down to 4.0 cm. The two highest germinations occurred at 1.0 (12 per cent) and 2.0 cm. (12 per cent). At this time the flats were again permitted to dry out thoroughly for another 3-week period. Counts 14 days later in this third period disclosed very little germination. The soil by this time had become caked over with an algal growth and this together with greater soil compaction may have influenced further germination by restricting aeration. Thus the total germination in this test was 42 per cent at the 2.0 cm. depth. Perhaps by further periods of prolonged drying, additional seeds might be forced to germinate. Just how long such dormant seeds might remain viable is not known, but an indication may be obtained from the well known experiments of Dr. W. J. Beal at Michigan State College on the related species, *S. lutescens*. Seeds of this species germinated after 30 years of burial (4), but not after 35 years, or at any of the test periods thereafter (10).

In the chemical soil treatment test described later (Table V) the pots were allowed to dry out for a 21-day period and they were then watered normally again. Germination of 12 to 16 per cent occurred after a 14-day wetting period in two of the treatments, Crag Hercibide 1 and 2,4-D. The largest germination for the entire period of 56 days, including the drying out period, totaled only 40 per cent. Thus, with a maximum possible germination of 80 per cent, 40 per cent of the seed were still dormant and presumably capable of germination following additional drying out periods.

An additional experiment was set up in which seeds cultured on moist filter papers in Petri dishes were subjected to a daily alternation of moist and dry conditions throughout an 8-day period. At the end of this period one set of 100 seed of this series was kept on the same filter papers, and cultured in the dark at 26° C. and a second set was transferred to soil in 4-inch clay pots and grown in the greenhouse. The results after 14 days showed a germination value of 26 and 54 per cent respectively. The controls on filter paper and in soil maintained for approximately the same period of time gave values of 13 and 40 per cent respectively. This cycle of alternate wetting and drying appreciably increased the germination both in the Petri dish and the soil series, and no marked injury to the seeds occurred. It is not known whether a longer period of treatment would produce the maximum germination of about 80 per cent. It is just possible that some injury might occur, thus the maximum germination of 80 per cent would never be reached.

Griswold (20) found that alternate wetting and drying of seeds of western range plants also promoted better germination for some of the species, while in certain others it did not. Other workers (5, 6, 9, 36) have observed the importance of alternating moisture, loosening of the soil, and certain oxygen levels as important factors in favoring the germination of weed seeds.

Beal (4) in his classic studies on the viability of seeds found that if he permitted the soil of the test flats to dry out further germination of the weed seeds occurred.

Germination inhibitors. From the data presented above on both of these alternate wetting and drying experiments, it would appear that one of the factors involved here is the absorption of inhibitory substances from the seeds by filter papers or by the surrounding soil as the moisture moves from the fully imbibed seed during the drying out process. A temperature factor also exists under field conditions since a moist surface layer of soil would be cooler than a dry surface layer. However, in the Petri dish experiment the temperature was held constant at 21° C. In this case the porous filter paper with its great surface area would roughly correspond with porous soil of vastly greater surface area.

The role of germination inhibitors has been extensively explored by several investigators (3, 12). Using the filter paper absorption technique there were indications of inhibitors present in giant foxtail seeds. A lot of 2 g. of seed was grown on moist filter paper at 21° C. for 3 days, after which this lot of seed was discarded, and a fresh lot of 50 seeds was placed on the same paper and grown under the same conditions for 14 days. Germination at this time was 6 per cent, with a value of 24 per cent for the control. This test was repeated with the use of several different weights of seed, and the results showed that 2 g. of seed was the minimum amount to give inhibition under these conditions.

Further work should be done with these inhibitors since it is quite likely these substances are in part responsible for the irregular or periodic germination of weed seeds. It also is quite possible that such results as reported by Gianfagna and Pridham (18) for the irregular crabgrass germination might be explained in part by the removal of germination inhibiting substances by the alternate wetting and drying processes of the surface soil.

CHEMICAL SOIL TREATMENT TESTS

The application of germinative toxicants (30) to the recently tilled soil surface generally will prevent the establishment of weed seedlings. If no crop is involved the number of chemicals available for use is quite large. However, when treatment of an established crop is contemplated the selection of the appropriate chemical is a more difficult one. Since the giant foxtail is a problem weed in corn, soybeans, and young pastures, a special chemical might well be required in each of these crops. A series of tests was initiated to see which of five available chemicals might be the most effective germinative toxicant.

The triethanolamine salt of 2,4-dichlorophenoxyacetic acid (2,4-D),

Crag Herbicide 1[2] [sodium 2-(2,4-dichlorophenoxy)ethyl sulfate] (31), chloro-IPC[3] [*O*-isopropyl-*N*-(3-chlorophenyl) carbamate] (45), dichloral urea[2] (29), and CMU (*p*-chlorophenyl dimethyl urea)[4] (8) were tested at rates of 2.5, 5, 10, and 25 lb. per acre. Two replicate 4-inch clay pots each containing 25 giant foxtail seeds were prepared, watered, and allowed to stand for about three hours. Aqueous preparations of the various chemicals were then applied in a volume of 40 ml. to each of the pots.

After 21 days of culture under average greenhouse conditions the pots were allowed to dry out for 21 days after which period of time they were again watered as formerly for a 14-day period (56 days after the initial planting). This procedure would disclose any possible injury to the seeds that were dormant at the time of the initial chemical application. Germination data taken at 7, 14, and 21 days, and after a period of drying (56 days) are presented in Table V with illustrations of some of these tests shown in Figure 3.

Crag Herbicide 1 and 2,4-D were about equally effective at rates of 2.5 and 5 lb. per acre, giving roughly about 50 per cent reduction in germination, and after drying the germination range 6 to 10 per cent was similar to the controls. However, at 10 and 25 lb. per acre Crag Herbicide 1 appeared superior to 2,4-D with a germination value of 4 per cent and 0 per cent respectively for the former, and 22 and 10 per cent respectively for the latter. Germination after the drying period at these two higher rates still disclosed germination within close range of the controls, but yet some reduction at the 25 lb. per acre rate.

Chloro-IPC and dichloral urea are principally grass toxicants and hence were quite effective in controlling giant foxtail. Chloro-IPC was somewhat better giving complete control at 5, 10, and 25 lb. per acre; while a few seedlings (though dwarfed) did emerge at the 5 and 10 lb. rates, none did so at 25 lb. per acre. After a drying out period of 21 days, followed by a moist period of 14 days, seedlings again appeared in both of these series. Thus no permanent injury occurred to the dormant seeds.

CMU was quite effective, destroying the seedlings at all rates tested. However, after the drying out period, additional seedlings appeared indicating that no destruction of the dormant seeds occurred. This second growth of seedlings did not survive, however, indicating the chemical was still present in an active form.

The above three materials are all good grass toxicants and hence probably could not be used in cereal crops. There is some indication, however, that dichloral urea is not injurious to already emerged corn. It is possible that dichloral urea could be used in well established corn to control the

[2] Samples furnished by Carbide and Carbon Chemicals Co., New York, N. Y.

[3] Sample furnished by Pittsburgh Plate Glass Co., Pittsburgh, Pa.

[4] Sample furnished by E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.

FIGURE 3. Control of giant foxtail following applications of a series of four chemicals to the soil surface after planting of the seeds. (A) Crag Herbicide 1; (B) triethanolamine salt of 2,4-D; (C) chloro-IPC; (D) CMU. Rates from left to right in each case are: 0, 2.5, 5, 10, and 25 lb. per acre. (Photographed 30 days after treatment.)

TABLE V

EFFECT OF CHEMICAL SOIL TREATMENT AND SOIL DESICCATION UPON THE GERMINATION OF GIANT FOXTAIL SEED PLANTED IN CLAY POTS IN THE GREENHOUSE

Rate in lb. per acre	No. of days after planting	Average per cent germination of 50 seeds at the chemical treatment indicated				
		Crag Herbicide 1	2,4-D	Chloro-IPC	Dichloral urea	CMU
2.5	7	12	10	12	28	16
	14	18	14	24	32*	16
	21	16	14	26	42*	8
	56**	10	6	2		4
	Total	26	20	28		12
5.0	7	22	14	16	16	32
	14	28	22	22*	50*	28
	21	28	26	20*	54*	0
	56**	10	8	12		2
	Total	38	34	32		2
10.0	7	4	4	30*	30	12
	14	4	20	40*	40*	12
	21	4	22	38*	42*	0
	56**	16	12	0		0
	Total	20	34	38*		0
25.0	7	0	4	22*	16	14
	14	2	4	24*	52*	18
	21	0	10	22*	50*	0
	56**	2	6	4*		0
	Total	4	16	26*		0
Control†		A	B			
	7	28	30			
	14	30	60			
	21	32	64			
	56**	8				
	Total	40				

* Seedlings severely inhibited.
** After 21-day drying period followed by 14 days' normal moisture.
† Control A was tested at the same time as all other treatments except dichloral urea. Control B was tested at the same time as the latter treatment.

later germination of the giant foxtail. In contrast, chloro-IPC has given injury in corn plantings, especially as a preemergence application (45).

Because of lack of foliage injury from Crag Herbicide 1, applications could well be made to corn at various intervals following seedling emergence up to and including lay-by time. Under field conditions excellent control of crabgrass has been obtained with rates of 2 lb. per acre (31), and this rate or rates up to 5 lb. per acre should control the somewhat more deeply germinating giant foxtail. Since Crag Herbicide 1 produces no hormonal responses in plants from foliage applications, corn does not develop brittleness following sprays of this herbicide.

Another test of dichloral urea involved applying the chemical at a rate of 5 lb. per acre to a greenhouse flat which had been planted with giant foxtail seeds (3 g. per flat). The flats were planted on November 21, 1951, and the chemical was applied at a delivery rate of 100 gal. per acre on November 23, 1951. Control was excellent with dichloral urea. While the seeds did germinate, the seedlings were greatly stunted and they eventually died. Photographs of this experiment were taken on December 18, 1951, and are shown in Figure 2, B and C.

The question of the destruction of dormant seeds in the soil by chemicals has frequently been raised. Mitchell and Brown (37) concluded in their studies on *Trifolium subterraneum* that 2,4-D had no effect on dormant seeds of this species. Everson (14) concluded that 2,4-D penetrates the water-permeable seed coats of beans, corn, and embryo dormant giant ragweed, but not water-impermeable seeds of butterprint. Where 2,4-D effects are evident from treated water-impermeable seeds, the 2,4-D is present only in the seed coat. Thus embryo dormant water-permeable seed are more likely to be destroyed by chemical soil treatment than are the water-impermeable embryo dormant seeds.

FIELD APPLICATIONS

Many factors are involved in the irregular or periodic germination of weed seeds so widely observed in cultivated soils and elsewhere. With the newer methods of chemical weed control, particularly that of early preemergence or residual type preemergence, this problem of weed seed germination has assumed an even greater importance. Many methods, chemical and otherwise, are now used for the eradication of weeds, but it would appear that chemical applications for the prevention of weed seed germination are among those offering the greatest promise for control, with a minimum of crop injury. But the factor of seed dormancy and irregular germination has often been the basis for many of the reports of unsuccessful weed control by this chemical method. Still, the large number of reports of successful results have shown that under many conditions and with certain types of weeds this chemical method offers great prospects.

However, by understanding the environmental factors most conducive to germination and seedling development of the weeds to be controlled, the chemical methods at hand may be utilized most effectively. As disclosed in some of the experiments just described, the single factor of looseness or compaction of the soil has a considerable relation to the depth of germination, and hence to the effectiveness of chemical treatment. This would imply that in order to encourage only shallow germination of weeds, very loose soil should be compacted—either by the natural forces of rain, or by agricultural implements—prior to chemical treatment. In some perennial crops such as asparagus or nursery stock, soil cultivation should

be held to a minimum, and chemical applications made to the intact soil surface at frequent enough intervals to maintain a satisfactory level of chemical throughout the growing season.

With the use of certain chemicals depth of weed emergence may not always be critical. This would be especially true with such seedling toxicants as CMU and dichloral urea but not for the more strictly germinative seed toxicants such as 2,4-D and Crag Herbicide 1. The degree of residual action, and the specificity of the chemical—whether for grass or broad-leaved weeds—would also have a bearing upon this problem. The stage of weed seed germination is frequently more important than depth of emergence. Many investigators have shown that better weed control can be obtained by permitting a few days delay between fitting of the soil and chemical application. The problem of the dormant seed is ever present, however, and save for the arsenicals there are few chemicals that are effective for controlling dormant seed.

This single factor of dormancy in weed seeds is the chief cause of irregular germination. The present experiments and those reported by other workers indicate that germination inhibitors are at least partially responsible for this behavior. Evidence is offered to show that alternate wetting and drying of the soil, as well as the seed, stimulates germination. It is postulated that this process results in the removal of the water-soluble inhibiting substances from the seed. Thus alternate wetting and drying of an undisturbed surface soil, as in a lawn, may result in continued germination of such seed as crabgrass without seed renewal. Were it possible to maintain weed seeds in a permanent state of dormancy, another effective method of weed control might be possible.

SUMMARY

Petri dish tests revealed that the germination of giant foxtail seeds seldom exceeded 20 to 34 per cent. Scarification of the seeds decreased rather than increased germination. Germination was increased by culturing in association with soil, by treatment with potassium nitrate and sodium thiocyanate solutions, and by alternating temperatures (21° to 37° C.) and moist storage at 21° C. The presence of germination inhibitors in the seeds was indicated by means of filter paper absorption experiments. In compact soil giant foxtail seed germinated and emerged when planted no more deeply than 3 cm. while in looser well-aerated soil, they emerged from a depth of 12 cm. Alternate wetting and drying of the soil also stimulated further germination of seeds planted at several depths. It is suggested that the alternate wetting and drying process results in the partial removal, at least, of germination inhibiting substances from the seeds.

Chemical soil treatment studies with the triethanolamine salt of 2,4-D, Crag Herbicide 1, dichloral urea, and CMU disclosed that each of these is

effective in controlling the germination or seedling development of giant foxtail at several of the rates tested. It was suggested that Crag Herbicide 1 be used to control this weed in corn any time following emergence, up to and including lay-by and that dichloral urea applied at 5 lb. per acre might also be employed in well established corn plantings. Chloro-IPC and CMU were both quite effective, the latter checking seedling growth at rates as low as 2.5 lb. per acre. Alternate wetting and drying of this chemical soil treatment series disclosed that the chemicals did not prevent the dormant seed from germinating after normal cultural conditions were restored. In the CMU series, however, the seedlings did not survive, indicating residual action of the chemical.

LITERATURE CITED

1. ALLARD, H. A. Some plants found in northern Virginia and West Virginia. Virginia Jour. Sci. **2**: 116–119. 1941.
2. ARBER, AGNES. The Gramineae. A study of cereal, bamboo, and grass. 480 pp. Cambridge University Press, Cambridge. 1934.
3. BARTON, LELA V., and MARIE L. SOLT. Growth inhibitors in seeds. Contrib. Boyce Thompson Inst. **15**: 259–278. 1948.
4. BEAL, W. J. The vitality of seeds. Bot. Gaz. **40**: 140–143. 1905.
5. BIBBEY, R. O. The influence of environment upon the germination of weed seeds. Sci. Agric. **16**: 141–150. 1935.
6. ————. Physiological studies of weed seed germination. Plant Physiol. **23**: 467–484. 1948.
7. CHASE, AGNES. The meek that inherit the earth. *In* U. S. Dept. Agric. Yearbook. p. 8–15. 1948.
8. COWART, L. E. Some herbicidal properties of CMU. Fifth Proc. Southern Weed Control Conf. Atlanta, Ga., 1952. p. 10–11.
9. CROCKER, WILLIAM. Growth of plants—Twenty years' research at Boyce Thompson Institute. 459 pp. Reinhold Pub. Corp., New York. 1948.
10. DARLINGTON, H. T. The 50-year period for Dr. Beal's seed viability experiment. Amer. Jour. Bot. **18**: 262–265. 1931.
11. EVANS, MORGAN W. The life history of timothy. U. S. Dept. Agric. Bull. 1450. 55 pp. 1927.
12. EVENARI, MICHAEL. Germination inhibitors. Bot. Rev. **15**: 153–194. 1949.
13. EVERS, ROBERT A. *Setaria Faberii* in Illinois. Rhodora **51**: 391–392. 1949.
14. EVERSON, LEROY. Further studies on the effect of 2,4-D on seeds. Proc. Assoc. Off. Seed Anal. N. Amer. **40**: 84–87. 1950.
15. FABIAN, INGE. Beiträge zum Lang- und Kurztagsproblem. Zeitschr. Bot. **33**: 305–357. 1938.
16. FERNALD, M. L. *Setaria Faberii* in eastern America. Rhodora **46**: 57–58. 1944.
17. GARMAN, HELEN R., and LELA V. BARTON. Germination of seeds of *Panicum anceps* Michx. Contrib. Boyce Thompson Inst. **14**: 117–122. 1946.
18. GIANFAGNA, ALFRED J., and A. M. S. PRIDHAM. Some aspects of dormancy and germination of crabgrass seed, *Digitaria sanguinalis* Scop. Proc. Amer. Soc. Hort. Sci. **58**: 291–297. 1951.
19. Gray's Manual of Botany. 8th ed., rev. by Merritt Lyndon Fernald. 1632 pp. American Book Co., New York. 1950.
20. GRISWOLD, SYLVIA M. Effect of alternate moistening and drying on germination of seeds of western range plants. Bot. Gaz. **98**: 243–269. 1936.

21. HANF, M. Keimung und Entwicklung des Klettenlabkrautes (*Galium aparine* L.) in verschiedener Aussaattiefe. Angew. Bot. **23**: 152–163. 1941.
22. ————. Bewurzelung und Wuchs von Unkrautkeimlingen in verschiedenen Böden. Jour. Landw. **90**: 125–146. 1943.
23. ————. Keimung von Unkräutern unter verschiedenen Bedingungen im Boden. Landw. Jahrb. **93**: 169–259. 1944. (*Abstr. in* Biol. Abstr. **24**: 33902. 1950.)
24. HEISE, A. C. Germination of green foxtail seeds. Proc. Assoc. Off. Seed Anal. N. Amer. **33**: 43–44. 1941.
25. HERRMANN, WOLFGANG. Ueber das phylogenetische Alter des mechanischen Gewebesystems bei *Setaria*. Beitr. Biol. Pflanzen **10**: 1–69. 1911.
26. HITCHCOCK, A. S. Manual of the grasses of the United States. 2nd ed., rev. by Agnes Chase. U. S. Dept. Agric. Misc. Publ. 200. 1051 pp. 1950 [1951].
27. JOHNSON, L. P. V. General preliminary studies on the physiology of delayed germination in *Avena fatua*. Canadian Jour. Res. Sec. C. **13**: 283–300. 1935.
28. KEYS, CHARLES E. Observations on the seed and germination of *Setaria italica* (L.) Beauv. Trans. Kansas Acad. Sci. **52**: 474–477. 1949.
29. KING, LAWRENCE J. Dichloral urea (Experimental Herbicide 2) and sodium 2,4-dichlorophenoxyethyl sulfate (Experimental Herbicide 1) as selective herbicides. Proc. Northeastern States Weed Control Conf. New York City, Jan. 1950. p. 302–309.
30. KING, L. J., and J. A. LAMBRECH. A new chemical for use in pre-emergence weeding. Proc. Northeastern States Weed Control Conf. New York City, Jan. 1949. p. 34–36.
31. KING, LAWRENCE J., J. A. LAMBRECH, and THOMAS P. FINN. Herbicidal properties of sodium 2,4-dichlorophenoxyethyl sulfate. Contrib. Boyce Thompson Inst. **16**: 191–208. 1950.
32. KIRK, L. E., and T. K. PAVLYCHENKO. Vegetative propagation of wild oats, *Avena fatua*, and other economically important species of Aveneae and Hordeae. Canadian Jour. Res. **7**: 204–220. 1932.
33. KISHIMOTO, ENKO. Chromosomenzahlen in den Gattungen *Panicum* und *Setaria*. I. Chromosomenzahlen einiger *Setaria*-Arten. Cytologia **9**: 23–27. 1937.
34. KOLK, HELMUT. Gronigsbiologiska studier på ogräsarter. (Studies in germination biology in weeds.) Publ. Inst. Plant Husbandry Royal Agric. Coll. Sweden **2**: 108–167. (English summ.) 1947.
35. KRÜGER, LEO. Ein Beitrag zur Biologie von *Chenopodium album*. Bedeutung und Bekämpfung als landwirtschaftliches Unkraut. Angew. Bot. **13**: 1–121. 1931.
36. MARTIN, JOHN N. Germination studies of the seeds of some common weeds. Proc. Iowa Acad. Sci. **50**: 221–228. 1943.
37. MITCHELL, JOHN W., and JAMES W. BROWN. Relative sensitivity of dormant and germinating seeds to 2,4-D. Science **106**: 266–267. 1947.
38. MUSIL, ALBINA F. Some brief notes on the identification of seeds of foxtail millet, *Setaria italica*. Proc. Assoc. Off. Seed Anal. N. Amer. **40**: 55–57. 1950.
39. PAMMEL, L. H., and CHARLOTTE M. KING. Results of seed investigations for 1908 and 1909. Iowa Agric. Exp. Sta. Bull. 115: 154–175. 1910.
40. POHL, RICHARD W. The genus *Setaria* in Iowa. Iowa State Coll. Jour. Sci. **25**: 501–508. 1951.
41. READ, Hadley. New weed threatens cornbelt. Successful Farming. **49**(6): 94, 96–97. June, 1951.
42. SALISBURY, E. J. The reproductive capacity of plants. 244 pp. G. Bell and Sons, Ltd., London. 1942.
43. SCOTT, Walter O. Watch out for giant foxtail. Farm Jour. **75**(8): 40. Aug., 1951.
44. SLIFE, F. W., and W. O. SCOTT. Giant foxtail. New cornbelt weed menace. Crops and Soils **3**(7): 22–23. 1951.

45. STEVENS, L. F., and R. F. CARLSON. The effects of chloro IPC on various crops and its residual properties in various soils. Proc. Northeastern States Weed Control Conf. 6th ann. meeting, New York City. Jan. 1952. p. 33–34.
46. STEVENS, O. A. The number and weight of seeds produced by weeds. Amer. Jour. Bot. **19**: 784–794. 1932.
47. STONE, A. L. The weed content of seeds. Proc. Assoc. Off. Seed Anal. N. Amer. **1914**: 23–27. 1915.
48. THURSTON, JOAN M. Some experiments and field observations on the germination of the wild oat (*Avena fatua* and *A. ludoviciana*) seeds in soil and the emergence of seedlings. Ann. App. Biol. **38**: 812–832. 1951.
49. WALDRON, L. R. Weed studies. Vitality and growth of buried weed seed. North Dakota Agric. Exp. Sta. Bull. **62**: 439–446. 1904.
50. WARINGTON, KATHERINE. The effect of constant and fluctuating temperature on the germination of the weed seeds in arable soil. Jour. Ecol. **24**: 185–204. 1936.
51. WOOD, CARROLL E., JR. *Setaria Faberii* in North Carolina. Rhodora **48**: 391–392. 1946

INDEX

Aedes aegypti: insecticidal activity tests on, 453
Alligator weed: See *Alternanthera philoxeroides*
Alternanthera philoxeroides: control by 2,4-dichlorophenoxyacetic acid, 103; control with ground equipment, 111; control with aerial equipment, 116; growth and reproduction, 91
Anethum graveolens: effect of *Lygus oblineatus* on, 280
Antibiotics: introduction into *Nicotiana tabacum* by wick method, 398; tested *in vitro* on tobacco-mosaic virus, 398
Apple trees: See *Pyrus malus*
Avena sativa: effect of seed soaking, 55

Bacillus cereus var. *mycoides:* activator of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate, 436
Back, R. C. The insecticidal activity of the eta isomer of 1,2,3,4,5,6-hexachlorocyclohexane, 451
Bacteria, sulfate-reducing: formation of metal sulfides, 85; growth in presence of metal sulfides, 87
Barton, Lela V. Germination of seeds of *Juniperus virginiana* L., 387; Relation of different gases to the soaking injury of seeds, 55
Barton, Lela V., and A. Goenaga. An electrical moisture meter for the determination of moisture in coffee beans, 461
Barton, Lela V., and Janet E. Hotchkiss. Germination of seeds of *Eichhornia crassipes* Solms., 215
Beale, Helen Purdy, and Carolyn R. Jones. Virus diseases of tobacco mosaic and potato yellow dwarf not controlled by certain purified antibiotics, 395
Benzoic acid: chlorosubstituted, chemical structure and physiological activity, 209; substituted, growth regulators, 209, 419
Benzoquinones: bacteriostasis, 361; effect on enzyme systems, 362
Blatella germanica: effects of irregular sex ratios on growth response data from nutritional assays, 455; insecticidal activity tests upon, 453; relation of sex to weight, 456; weight differences between sexes on control diet, 456
Burchfield, Harry P., and George L. McNew. Mechanism of particle size effects of fungicides on plant protection, 131
Burchfield, Harry P.: See also McNew, George L.

Carbon dioxide: effect on soaking injury of seeds, 55
Carroll, Robert B. Activation of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate, 409
Chandler, Clyde. Flowering habits and fertility of some *Cinchona* species in Guatemala, 249
Chimaera: account for new types, 221, 224
Chloranil: fungicidal activity, 359
Chloro-IPC: See *O*-Isopropyl *N*-(3-chlorophenyl)carbamate
p-Chlorophenyl dimethyl urea: as germinative toxicant for *Setaria Faberii*, 480
Chromate complexes: as fungicides, 375; as seed protectants, 5; mammalian toxicology, 7; solubility behavior, 375
Cinchona: breeding, 253
Cinchona Calisaya: flowering habits, 252
Cinchona Ledgeriana: flowering habits, 251
Cinchona succirubra: flowering habits, 252
CMU: See *p*-Chlorophenyl dimethyl urea
Cocklebur: See *Xanthium canadense*
Cockroach: See *Blattella germanica*
Coffee in "coco": adjustment of moisture content in samples, 462; determination of moisture content, 462
Colchicine: seeds and seedlings treated with, 257
Copper-zinc-chromate complex: as a seed protectant, 6; development, 381
Cotton, Delta Pine: See *Gossypium hirsutum*
Crag Herbicide 1: See Sodium 2-(2,4-dichlorophenoxy)ethyl sulfate
Crocker, William: The man and scientist (January 27, 1874-February 11, 1950), 1
Cucumber: See *Cucurbita sativus*
Cucurbita sativus: inhibition of root elongation, 410
Cupric γ-chloroacetoacetanilide as a seed protectant, 6

Depth of planting: effect on germination of *Setaria italica*, 477
Desulfovibrio desulfuricans: See Bacteria, sulfate-reducing

Dichloral urea: as germinative toxicant for *Setaria Faberii*, 480
2,3-Dichloro-1,4-naphthoquinone: effect of particle size, 140; fungicidal action, 363
2,6-Dichloro-3-nitrobenzoic acid: growth regulation in *Lycopersicon esculentum*, 420
2,6-Dichlorobenzaldehyde: growth-regulating effects in *Lycopersicon esculentum*, 420
2,6-Dichlorobenzoic acid: growth regulation in *Lycopersicon esculentum*, 420
Dichloronaphthoquinone: particle size in relation to its fungitoxicity, 163
2,4-Dichlorophenoxyacetic acid: as control for *Alternanthera philoxeroides*, 103; as control for *Eichhornia crassipes*, 94; biological tests for detection, 439; leaf modification caused by sub-lethal doses, 446; quantitative method of measuring plant responses, 225
2,4-Dichlorophenoxyacetic acid, polyethylene glycol esters of: herbicidal properties, 267
2,4-Dichlorophenoxyacetic acid, polypropylene glycol esters of: herbicidal properties, 267
2,4-Dichlorophenoxyacetic acid, triethanolamine salt of: as toxicant for seed of *Setaria Faberii*, 480
2,4-Dichlorophenoxyethanol: possible mechanism of action as growth regulator, 413; relation to growth inhibition, 410
Dill: See *Anethum graveolens*
4,5-Dimethyl-2-mercaptothiazole: fungitoxicity of some derivatives, 261
Dimethyldithiocarbamate and sulfur dichloride: reaction product of, as a seed protectant, 6
Disodium ethylene bisdithiocarbamate: vegetable blight control, 308
Dithiocarbamic acid derivatives as fungicides, 303
DUNN, E. E.: See GRUENHAGEN, R. H.
Dust deposition in vacuum bell jar duster, 29

EARLE, T. T.: See HITCHCOCK, A. E.
Eichhornia crassipes: control by 2,4-dichlorophenoxyacetic acid, 94; control with aerial equipment, 116; control with ground equipment, 111; germination of seeds, 215; growth and reproduction, 91, 122
Electrical moisture tester (Universal): reliability in testing moisture content of coffee in "coco," 463
Embryolessness: See Seeds
Embryos, excised: effect of gases during soaking, 64
Enzyme systems: effect of benzoquinones, 362; effect of naphthoquinones, 366

Fennel: See *Foeniculum dulce*
Ferbam: See Ferric dimethyldithiocarbamate
Ferric dimethyldithiocarbamate: fruit disease control, 306
FINN, THOMAS P.: See KING, LAWRENCE J.
FLEMION, FLORENCE, and BETTY TRAVIS MACNEAR. Reduction of vegetative growth and seed yield in umbelliferous plants by *Lygus oblineatus*, 279
FLEMION, FLORENCE, LAWRENCE P. MILLER, and RICHARD M. WEED. An estimate of the quantity of oral secretion deposited by *Lygus* when feeding on bean tissue, 429
FLEMION, FLORENCE, and JUNE OLSON. *Lygus* bugs in relation to seed production and occurrence of embryoless seeds in various umbelliferous species, 39
FLEMION, FLORENCE, RICHARD M. WEED, and LAWRENCE P. MILLER. Deposition of P^{32} into host tissue through the oral secretions of *Lygus oblineatus*, 285
FLENNER, A. L.: See TISDALE, W. H.
Flour beetle: See *Tribolium confusum*
Flower bud initiation: induced by photoperiods, 178
Foeniculum dulce: effect of *Lygus oblineatus*, 280
Fungicidal action: mechanism of, 131; theory of mechanism of dithiocarbamic acid derivatives, 308; theory of mechanism of some heterocyclic nitrogen compounds, 326
Fungicidal activity: benzoquinones, 359; chromate complexes, 375; influence of chemical structure, 261; mechanism of action of phenols, 355; naphthoquinones, 363
Fungicidal persistence as affected by particle size, 143
Fungicides: economic aspects, 295; techniques in testing, 27, 300

Fungitoxicity of dichloronaphthoquinone in diverse formulations, 166

Germination inhibitors, 479
Germination of seeds: effect of alternate wetting and drying, 477; effect of chemical soil treatments on *Setaria Faberii*, 479; effect of gases during soaking, 56; effect of planting depth, 472; effect of sodium 2,4-dichlorophenoxyacetate in soil, 196; effect of sodium 2,4-dichlorophenoxyethyl sulfate in soil, 196; effect of storage conditions on *Eichhornia crassipes*, 218; effect of temperature on *Setaria Faberii*, 472; requirement for *Juniperus virginiana*, 387
Giant foxtail grass: See *Setaria Faberii*
Glycine max: photoperiodic treatment, 185
Glyoxalidine derivatives: factors influencing spray injury to *Pyrus malus*, 21
GOENAGA, A.: See BARTON, LELA V.
Gossypium hirsutum: effect of diverse concentrations of 2,4-dichlorophenoxyacetic acid, 443
Growth, vegetative: reduction of, by *Lygus oblineatus*, 280
Growth regulators: application of two to same plant, 241; evaluation of individual responses to 2,4-dichlorophenoxyacetic acid by *Lycopersicon esculentum*, 232; quantitative method of measuring response of plants, 225; statistical procedures for evaluating induced responses, 226; substituted benzoic acids, 420
GRUENHAGEN, R. H., P. A. WOLF, and E. E. DUNN. Phenolic fungicides in agriculture and industry, 349

HARRY, J. B.: See WHALEY, F. R.
HARTZELL, ALBERT, and ELEANOR E. STORRS. Bioassay of insecticide spray residues in processed food, 47
Helianthus annuus: effect of seed soaking, 55
Helicopter: use for weed control in large areas, 119
2-Heptadecenylglyoxalidine: factors influencing spray injury to *Pyrus malus*, 22
2-Heptadecylglyoxalidine: factors influencing spray injury to *Pyrus malus*, 22
Heterocyclic nitrogen compounds: effect on fungitoxicity, 313
Heterostyly, 251
1,2,3,4,5,6-Hexachlorocyclohexane: insecticidal activity of isomers, 451
HILCHEY, JOHN D., and ROBERT L. PATTON. Effects of irregular sex ratios on growth response data from nutritional assays upon *Blattella germanica* (L.), 455
HITCHCOCK, A. E., and P. W. ZIMMERMAN. A quantitative method of measuring response of plants to growth regulators, 225
HITCHCOCK, A. E., P. W. ZIMMERMAN, HENRY KIRKPATRICK, JR., and T. T. EARLE. Growth and reproduction of water hyacinth and alligator weed and their control by means of 2,4-D, 91
HITCHCOCK, A. E.: See also ZIMMERMAN, P. W.
Hordeum vulgare: effect of seed soaking, 55
HORSFALL, JAMES G., and SAUL RICH. Fungitoxicity of heterocyclic nitrogen compounds, 313
HOTCHKISS, JANET E.: See BARTON, LELA V.
Housefly: See *Musca domestica*
Hydrogen sulfide: tolerance of sulfate-reducing bacteria, 74
Hydrogen-ion concentration: effect on activation of sodium 2-(2,4-dichlorophenoxy)-ethyl sulfate, 409

Insect nutrition: *Blattella germanica*, 455
Insecticide spray residues: bioassay of, in processed foods, 47
Insecticides: accidental contamination with 2,4-dichlorophenoxyacetic acid, method for detecting, 439
Insects, oral secretions: See *Lygus oblineatus*
O-Isopropyl *N*-(3-chlorophenyl)carbamate: as germinative toxicant for *Setaria Faberii*, 480

JONES, CAROLYN R.: See BEALE, HELEN PURDY
Juniperus virginiana: germination of seeds of, 387

KING, LAWRENCE J. Germination and chemical control of the giant foxtail grass, 469
KING, LAWRENCE J., and JOHN A. KRAMER, JR. Studies on the herbicidal properties and volatility of some polyethylene and polypropylene glycol esters of 2,4-D and 2,4,5-T, 267

KING, LAWRENCE J., J. A. LAMBRECH, and THOMAS P. FINN. Herbicidal properties of sodium 2,4-dichlorophenoxyethyl sulfate, 191
KIRKPATRICK, HENRY, JR.: See HITCHCOCK, A. E.; ZIMMERMAN, P. W.
KJENNERUD, JULIE: See ZIMMERMAN, P. W.
KRAMER, JOHN A., JR.: See KING, LAWRENCE J.

LAMBRECH, J. A.: See KING, LAWRENCE J.
Lycopersicon esculentum: growth regulation by substituted benzoic acids, 420; growth responses induced by chemicals other than 2,4-dichlorophenoxyacetic acid, 442; known responses to concentrations of 2,4-dichlorophenoxyacetic acid, 441
Lygus oblineatus: effect on embryolessness, 43; effect on Umbelliferae seed yield, 282; effect on vegetative growth of Umbelliferae, 280; injection of radioactive oral secretions into host tissue, 288; quantity of radioactive oral secretion deposited when feeding on bean pod tissue, 432; technique for measuring oral secretion deposited during feeding, 429; Umbelliferae seed production, 40

MCCALLAN, S. E. A. Factors influencing deposition in the vacuum bell jar duster, 27; Factors influencing spray injury by glyoxalidine derivatives to potted apple trees, 21; Some chromate complexes and organic compounds as seed protectants, 5; Testing techniques, 299
MACNEAR, BETTY TRAVIS: See FLEMION, FLORENCE
MCNEW, GEORGE L., and HARRY P. BURCHFIELD. Fungitoxicity and biological activity of quinones, 357; Particle size in relation to the fungitoxicity of dichloronaphthoquinone, 163
MCNEW, GEORGE L.: See also BURCHFIELD, HARRY P.
Macrosporium sarcinaeforme: See *Stemphylium sarcinaeforme*
Mercury-zinc-chromate complex as a seed protectant, 6
Metal sulfides: formed by sulfate-reducing bacteria, 86
MILLER, LAWRENCE P. Formation of metal sulfides through the activities of sulfate-reducing bacteria, 85; Tolerance of sulfate-reducing bacteria to hydrogen sulfide, 73
MILLER, LAWRENCE P.: See also FLEMION, FLORENCE
Moisture tester, electrical: for determination of moisture content of coffee beans, 461
Mosquito: See *Aedes aegypti*
Musca domestica: insecticidal activity tests upon, 453

Nabam: See Disodium ethylene bisdithiocarbamate
Naphthoquinones: effect on animal life, 365; effect on bacteria, 365; effect on enzyme systems, 366; fungicidal activity, 363
Nitrogen: effect on soaking injury of seeds, 55

OLSON, JUNE: See FLEMION, FLORENCE
Oxygen: effect on soaking injury of seeds, 55

P^{32}: See Radioactive phosphorus
Particle size and fungitoxicity of dichloronaphthoquinone: relationship of, 171
Particle size of fungicides: effect on foliage protection, 132; effect on seed protectants, 148
PATTON, ROBERT L.: See HILCHEY, JOHN D.
2-Pentadecylglyoxalidine: factors influencing spray injury to *Pyrus malus*, 22
pH: See Hydrogen-ion concentration
Phaseolus: effects of seed soaking, 55
Phenolic fungicides: adaptability, 350
Phosphorus: radioautographs, 291
Photoperiodic treatment: effect of temperature on results, 182; influence on branching habit, 183; on *Glycine max*, 185; on *Piqueria trinervia*, 178; on *Xanthium canadense*, 186
Phygon: See 2,3-Dichloro-1,4-naphthoquinone
Piqueria trinervia: photoperiodic treatment, 178; translocation of substituted benzoic acids, 423
Pisum sativum: effect of seed soaking, 55
Plant protection: mechanism of, 131
Polyethylene glycol esters of 2,4-dichlorophenoxyacetic acid: herbicidal proterties, 267

Polypropylene glycol esters of 2,4-dichlorophenoxyacetic acid: herbicidal properties, 267
Polypropylene glycol esters of 2,4,5-trichlorophenoxyacetic acid: herbicidal properties, 267
Potato-yellow-dwarf virus: tests with antibiotics for control in *Nicotiana rustica*, 403
PRILL, EDWARD A.: See ZIMMERMAN, P. W.
Pyrus malus, potted: spray injury of glyoxalidine derivatives to, 21

Quinones: biological activity, 357; particle size and fungitoxicity, 171; plant protection, 132, 148; relation between chemical structure and fungitoxicity, 360, 364

Radioactive phosphorus: oral secretion transfer by *Lygus oblineatus*, 288; used to measure oral secretion deposited by *Lygus oblineatus* when feeding, 429
Radioautographs: phosphorus, 291
Riboflavin: use to counteract effects of chemicals on *Cucurbita sativus* root inhibition, 414
RICH, SAUL: See HORSFALL, JAMES G.
Roots, adventitious, 221
Rosaceae: new varieties from adventitious buds on roots, 221

SCHMITT, C. G. Comparison of a series of derivatives of 4,5-dimethyl-2-mercaptothiazole for fungicidal efficacy, 261
Sclerotinia fructicola: test fungus for toxicity of heterocyclic nitrogen compounds, 316
Secretions of insects, oral: See *Lygus oblineatus*
Seed protectants: chromate complexes, 5; organic compounds, 5
Seed toxicants: effect on *Setaria Faberii*, 480
Seed storage: effect of chromate complex protectants on, 17
Seed weights: in relation to optimum and maximum depths of emergence in soil, 474
Seeds: colchicine treatment of *Cinchona* seeds and seedlings, 257; effect of gases on soaking injury, 55; embryoless Umbelliferae, 39; production affected by *Lygus oblineatus*, 42; See also Germination of seeds
Self-sterility, 255
Setaria Faberii: chemical control, 469; effect of chemical soil treatment and soil desiccation on germination in clay pots in greenhouse, 482; effect of depth of planting and soil desiccation on germination in greenhouse flats, 475; effect of depth of planting on chemical control, 472; germination of, 471; physical characteristics, 469
Setaria italica: effect of depth of planting on germination, 477
SINNOTT, EDMUND W. William Crocker—the man and scientist (January 27, 1874–February 11, 1950), 1
Soaking of seeds: relation of different gases to soaking injury, 55
Sodium 2,4-dichlorophenoxyethyl sulfate: See Sodium 2-(2,4-dichlorophenoxy)ethyl sulfate
Sodium 2-(2,4-dichlorophenoxy)ethyl sulfate: activation of, 409; as germinative toxicant for *Setaria Faberii*, 480; conversion to active plant growth regulator by *Bacillus cereus* var. *mycoides*, 436; effect of sterilization on growth inhibition, 412; effect on foliage, 192; effect on germination of seeds, 196; herbicidal properties compared with 2,4-dichlorophenoxyacetic acid, 192; measurement of activity, 409; residual action in greenhouse tests, 197; translocation in *Lycopersicon esculentum*, 414
Soybean, Biloxi: See *Glycine max*
Spergon: See Tetrachloro-*p*-benzoquinone
Sports: rose, 221
Stemphylium sarcinaeforme: test fungus for toxicity of heterocyclic nitrogen compounds, 316
Stevia: See *Piqueria trinervia*
Storage of seeds: effect on germination of *Eichhornia crassipes*, 218
STORRS, ELEANOR E.: See HARTZELL, ALBERT
Structure, chemical: chlorosubstituted derivatives of benzoic acid, chemical structure and physiological activity, 209; influence on fungicidal activity, 261, 313, 357; substituted benzoic acids, chemical structure and physiological activity, 421, 423
Sulfate-reducing bacteria: tolerance to hydrogen sulfide, 73

Surface area of fungicides related to persistence, 147
Synergism: of chromate with copper and with zinc, 377

Tetrachloro-*p*-benzoquinone: fungicidal activity, 359
Tetramethylthiuramdisulfide: seed and soil disinfectant, 306
Thiram: See Tetramethylthiuramdisulfide
TISDALE, W. H., and A. L. FLENNER. Derivatives of dithiocarbamic acid as fungicides, 303
Tobacco-mosaic virus: tests with antibiotics for control in *Nicotiana glutinosa*, 400
Toxicity of insecticide spray residues in processed foods, 51
Translocation: substituted benzoic acids, 422; variation in growth regulators, 212
Tribolium confusum: insecticidal activity tests upon, 453
2,3,6-Trichlorobenzaldehyde: growth-regulating effects, 209
2,3,6-Trichlorobenzoic acid: growth-regulating effects, 210, 420
2,4,5-Trichlorophenoxyacetic acid: as control for *Alternanthera philoxeroides*, 103
2,4,5-Trichlorophenoxyacetic acid, polyethylene glycol esters of: herbicidal properties, 267
2,4,5-Trichlorophenoxyacetic acid, polypropylene glycol esters of: herbicidal properties, 267
Triticum aestivum: effect of seed soaking, 55
2,4-D: See 2,4-Dichlorophenoxyacetic acid
2,4,5-T: See 2,4,5-Trichlorophenoxyacetic acid

Umbelliferae: embryolessness caused by *Lygus oblineatus*, 39; reduction of vegetative growth, 280; seed yield reduction by *Lygus oblineatus*, 280

Vacuum bell jar duster: construction of, 27; method of applying dusts, 29
Vicia faba: effect of seed soaking, 55
VLITOS, A. J. Biological activation of sodium 2-(2,4-dichlorophenoxy)ethyl sulfate by *Bacillus cereus* var. *mycoides*, 435

Water hyacinth: See *Eichhornia crassipes*
WEED, RICHARD M.: See FLEMION, FLORENCE
Weed control: by sodium 2,4-dichlorophenoxyethyl sulfate, 191; in *Asparagus officinalis* by 2,4-dichlorophenoxyethyl sulfate, 203; in sweet corn by 2,4-dichlorophenoxyethyl sulfate, 200
Weeds, common: optimum and maximum depths of emergence in soil, 474
WELLMAN, R. H.: The economics of using fungicides, 295
WHALEY, F. R., and J. B. HARRY. Chromate complexes as fungicides, 375
Wick method: introduction of chemicals into plants, 396
WOLF, P. A.: See GRUENHAGEN, R. H.

Xanthium canadense: effect of seed soaking, 55

Zea mays: effect of seed soaking, 55
ZIMMERMAN, P. W., and A. E. HITCHCOCK. Growth-regulating effects of chlorosubstituted derivatives of benzoic acid, 209; Rose "sports" from adventitious buds, 221
ZIMMERMAN, P. W., A. E. HITCHCOCK, and HENRY KIRKPATRICK, JR. Detection and determination of 2,4-D as a contaminant by biological methods, 439
ZIMMERMAN, P. W., A. E. HITCHCOCK, and EDWARD A. PRILL. Substituted benzoic acids as growth regulators, 419
ZIMMERMAN, P. W., and JULIE KJENNERUD. Flowering and other responses induced in *Piqueria trinervia* with photoperiodic treatment, 177
ZIMMERMAN, P. W.: See also HITCHCOCK, A. E.
Zinc dimethyldithiocarbamate: vegetable crop disease control, 307
Zinc ethylene bisdithiocarbamate: vegetable blight control, 308
Zineb: See Zinc ethylene bisdithiocarbamate
Ziram: See Zinc dimethyldithiocarbamate